

**NUCLEIC ACID MOLECULES AND POLYPEPTIDES  
FOR A HUMAN CATION CHANNEL POLYPEPTIDE**

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1. FIELD OF THE INVENTION

The present invention relates to the isolation and identification of human nucleic acid molecules and proteins and polypeptides encoded by such nucleic acid molecules, or degenerate variants thereof, encoding a human cyclic nucleotide gated (CNG) cation channel. The proteins and polypeptides of the invention represent a novel cation channel that may be a therapeutically valuable target for drug delivery in the treatment of human diseases that involve calcium, sodium, potassium or other ionic homeostatic dysfunction, such as central nervous system (CNS) disorders, e.g., stroke, anxiety and depression, or degenerative neurological disorders such as Alzheimer's disease or Parkinson's disease, or other disorders such as cardiac disorders, e.g., arrhythmia, diabetes, chronic pain, hypercalcemia, hypocalcemia, hypercalciuria, hypocalciuria, or ion disorders associated with immunological disorders, gastro-intestinal (GI) tract disorders, or renal or liver disease. Moreover, the polypeptides of the present invention can function as effector molecules, reflecting the intracellular concentration of cAMP and/or cGMP. Accordingly the present invention also relates to the use of the CNG cation channel polypeptides disclosed herein for the detection of modulators of intracellular cAMP and/or cGMP levels. More specifically, the present invention relates to the use of CNG cation channel



polypeptides as components of assays for the detection of  
 5 antagonists and/or agonists of G-protein coupled receptor  
 activity, which may be therapeutically useful molecules.

## 2. BACKGROUND OF THE INVENTION

Control of the internal ionic environment is an  
 10 extremely important function of all living cells. Ion  
 exchange with the external medium is regulated by a  
 variety of means, the most important of which are various  
 transporters and ion channels. Ion channels in particular  
 have been important targets for the development of  
 15 therapeutic compounds in the treatment of disease.

A number of proteins have been described as forming  
 ion channels. Among these are proteins that have been  
 shown to function as cation channels of varying degrees  
 of selectivity and with different, and in some cases  
 20 unknown, mechanisms for channel gating. Within the family  
 of cation channels, there is an identified group that  
 includes cyclic nucleotide gated (CNG) channels, which  
 are activated by intracellular binding of cAMP and/or  
 cGMP to CNG polypeptides. CNG channels are nonselective  
 25 cation channels which allow the passage of monovalent  
 cations, including both  $K^+$  and  $Na^+$  ions, as well as  
 divalent cations. Although CNG channels can transport  
 both monovalent and divalent cations,  $Ca^{+2}$  blocks the flow  
 of monovalent cations through the channel (Zagotta et al.  
 30 1996 Ann. Rev. Neurosci. 19: 235-63). CNG channels were  
 originally found to be involved in signal transduction  
 within sensory tissues.

The first cDNA clone encoding a CNG channel  
 $\alpha$ -subunit polypeptide was isolated from bovine rod tissue  
 35 (Kaupp et al. 1989 Nature 342: 762-66). Subsequently, a  
 series of CNG  $\alpha$ -subunit polypeptide encoding genes were  
 isolated from other tissues and species that encoded

- proteins structurally related to the bovine rod CNG  $\alpha$ -subunit polypeptide. (Bauman et al. 1994 EMBO J 13:5040-50; Biel et al. 1993 FEBS Lett 329: 134-38; Biel et al. 1994 Proc. Natl. Acad. Sci. USA 91:3505-09; Bönigk et al. 1993 Neuron 10: 865-77; Bradley et al. 1994 Proc. Natl. Acad. Sci. USA 91: 8890-94; Chen et al. 1993 Nature 362: 764-67; Dhallan et al. 1990 Nature 347: 184-87; Dhallan et al. 1992 J. Neurosci. 12:3248-56; Goulding et al. 1992 Neuron 8: 45-58; Liman et al. 1994 Neuron 13: 611-21; Ludwig et al. 1990 FEBS Lett. 270: 24-29; Weyland et al. 1994 Nature 368: 859-63). Although these genes were shown to be structurally related, different tissue-specific and species-specific expression of those genes was established (Distler et al. 1994 Neuropharmacology 33: 1275-82). For example, the full-length cDNA encoding the CNG channel polypeptide isolated from rabbit aorta was reported to be 93.7% homologous with bovine olfactory CNG polypeptide (Biel et al. 1993 FEBS Lett 329: 134-38). The functional role of the murine olfactory CNG polypeptide was established, *in vivo*, by constructing knockout mice lacking this gene. In these mutant mice, electrophysiological assays demonstrated that excitatory responses to odorants were undetectable, providing direct evidence for the role of this CNG channel in excitatory olfactory signal transduction (Brunet et al. 1996 Neuron 17: 682-93).
- A second, distinct cDNA clone encoding a CNG channel  $\alpha$ -subunit polypeptide was isolated initially from olfactory tissue (Dhallan et al. 1990 Nature 347: 184-87; Goulding et al. 1992 Neuron 8: 45-58; Ludwig et al. 1990 FEBS Lett. 270: 24-29) and later from rabbit aortic tissue (Biel et al. 1993 FEBS Lett. 329:134-38).

A third distinguishable cDNA clone encoding a CNG channel  $\alpha$ -subunit polypeptide has also been cloned from

- both sensory and non-sensory tissues: cone photoreceptors
- 5 (Bönigk et al. 1993 Neuron 10: 865-77), testis (Weyland et al. 1994 Nature 368: 859-63), and kidney tissue (Biel et al. 1994 Proc. Natl. Acad. Sci. 91: 3505-09).

- Amino acid sequence comparisons between and among the encoded CNG  $\alpha$ -subunit polypeptides identified above,
- 10 as well observed regions of homology between these proteins and other ion channels polypeptides, have been used to construct a structural model for CNG  $\alpha$ -subunit proteins (Zagotta et al. 1996 Ann. Rev. Neurosci. 19: 235-63). In this model, both the N-terminal and
- 15 C-terminal sequences of CNG  $\alpha$ -subunit polypeptide are positioned within the cell, and the termini of the  $\alpha$ -subunit protein are separated by six transmembrane segments, designated S1 to S6 when viewed in the N-terminal to C-terminal direction. The peptide segment
- 20 spanning the region between S5 and S6 constitutes the surface of the pore through which cations are conducted. In addition, binding sites for  $\text{Ca}^{+2}$ -Calmodulin and cAMP and/or cGMP have been identified on the intracellular N-terminal and C-terminal peptide segments, respectively.
- 25 Heterologous expression of the above  $\alpha$ -subunit polypeptide encoding CNG genes alone in, for example, *Xenopus* oocytes, provides a functional ion channel.

- Clones have also been isolated that encode a second polypeptide subunit, referred to as the  $\beta$ -subunit
- 30 polypeptide, of CNG channels (Chen et al. 1993; Bradley et al. 1994; Liman et al. 1994). Hydropathicity analyses of the two identified  $\beta$ -subunit polypeptides and amino acid sequence comparisons indicate that the  $\beta$ -subunit polypeptides, like the  $\alpha$ -subunit polypeptides, consist of
- 35 cytoplasmic amino- and carboxyl-termini separated by six transmembrane segments, a binding site for cyclic nucleotides within the C-terminal, intracellular portion

of the protein, and an ion-conducting pore. Despite these  
 5 structural similarities, there is only about a 40% amino  
 acid sequence identity observed between the CNG  $\alpha$ -subunit  
 and  $\beta$ -subunit polypeptides, in contrast to the  
 approximately 65% amino acid identity observed between  
 the various CNG  $\alpha$ -subunit polypeptide sequences.  
 10 Furthermore, and in contrast to the results obtained with  
 the  $\alpha$ -subunit CNG polypeptide, heterologous expression of  
 the  $\beta$ -subunit CNG polypeptide alone does not provide a  
 functional ion channel. However, co-expression of both a  
 and  $\beta$  CNG subunits yields heteromeric complexes having  
 15 properties exhibited by naturally-occurring CNG channels  
 that are not observed with homomeric CNG complexes formed  
 with the  $\alpha$ -subunit alone, including an increased affinity  
 for cyclic-nucleotide binding. The  $\beta$ -subunit CNG  
 polypeptides have, therefore, been referred to as  
 20 modulatory subunits of CNG channels (Biel et al. 1999,  
 Reviews of Physiology Biochemistry and Pharmacology 135:  
 151-71). Therefore, CNG channels consist of complexes of  
 homologous but distinguishable  $\alpha$ -subunits and  $\beta$ -subunits.

Kinetic models have been proposed which correlate  
 25 cyclic nucleotide binding with CNG channel opening. In  
 one model, summarized by Zagotta et al. (Zagotta et al.  
 1996 Ann. Rev. Neurosci. 19: 235-63), addition of cyclic  
 nucleotides to four cooperative binding sites induces  
 allosteric, conformational changes which result in the  
 30 opening of the CNG channel. The existence of multiple,  
 cooperative cyclic nucleotide binding sites forms the  
 basis of the exquisite sensitivity of CNG channels to  
 variations in the intracellular concentration of cAMP  
 and/or cGMP.

35 Cyclic nucleotides serve as intracellular second  
 messengers involved in regulated gene expression in  
 response to extracellular signals. Such signals may be

initiated, for example, by ligand binding to a G-protein  
5 coupled receptor, inducing conformational changes leading  
to intracellular activation of adenylyl or guanylyl cyclase.  
Resulting increases in the concentration of cyclic  
nucleotides can activate and open CNG channels, providing  
an influx of monovalent and/or divalent cations, and  
10 particularly calcium ions which, in turn, are directly  
involved in many aspects of biochemical and genetic  
regulation. It is through this biochemical cascade that  
CNG channels function as effector molecules for  
intracellular signals generated, for example, by  
15 G-protein coupled receptors.

Therefore, CNG channels are critical mediators of  
the cyclic nucleotide response generated in signal  
transduction pathways. The distribution of CNG channels  
within olfactory, auditory, brain, testicular, kidney,  
20 cardiac, and central nervous system tissues, demonstrates  
that CNG channels are important components of many  
critical biological processes. As such, human CNG  
channels are important targets, per se, for therapeutic  
intervention. Furthermore, CNG channels are also useful  
25 tools, in their role as effector molecules, for  
reflecting the modulation of intracellular cyclic  
nucleotide levels. Accordingly, CNG channels may also be  
used in assay procedures and screening methods for  
detection of compounds that modulate processes,  
30 including, but not limited to ligand binding and signal  
generation by G-protein coupled receptors, that affect  
intracellular cyclic nucleotide levels.

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5 3. SUMMARY OF THE INVENTION

The present invention relates to the isolation and identification of nucleic acid molecules and proteins and polypeptides encoded by such nucleic acid molecules, or degenerate variants thereof, that participate in the formation or function of human ion channels. More specifically, the nucleic acid molecules of the invention include a novel human gene that encodes a protein or polypeptide involved in the formation or function of a novel cation channel.

15 According to one embodiment of the invention, a novel, complete human cDNA, termed HBMYCNG, and the amino acid sequence of its derived expressed protein, is disclosed.

The compositions of this invention include nucleic acid molecules, e.g., the HBMYCNG gene, including recombinant DNA molecules, cloned genes or degenerate variants thereof, especially naturally occurring variants, which encode the HBMYCNG gene product, and antibodies directed against that gene product or conserved variants or fragments thereof.

In particular, the compositions of the present invention include nucleic acid molecules (also referred to herein as "HBMYCNG nucleic acid molecules" or "HBMYCNG nucleic acids") which comprise the following sequences:

30 (a) nucleic acid sequences of the human HBMYCNG gene, e.g., as depicted in FIG. 1, and as deposited with the American Type Culture Collection (ATCC) as disclosed in Section 7, *infra*, as well as allelic variants and homologs thereof; (b) nucleic acid sequences that encode the HBMYCNG, gene product amino acid sequences, as depicted in FIG. 2; (c) nucleic acid sequences of a variant of the human HBMYCNG gene, e.g., as depicted in

FIG. 5, and as deposited with the American Type Culture  
5 Collection (ATCC) as disclosed in Section 7, *infra*, as  
well as allelic variants and homologs thereof; (d)  
nucleic acid sequences that encode the variant HBMYCNG,  
gene product amino acid sequences, as depicted in FIG. 6;  
(e) nucleic acid sequences that encode portions of the  
10 HBMYCNG, gene product corresponding to functional domains  
and individual exons; (f) nucleic acid sequences  
comprising the novel complete gene sequence disclosed  
herein, or portions thereof, that encode mutants of the  
corresponding gene product in which all or a part of one  
15 or more of the domains is deleted or altered; (g) nucleic  
acid sequences that encode fusion proteins comprising the  
HBMYCNG gene product, or one or more of its domains,  
fused to a heterologous polypeptide; (h) nucleic acid  
sequences within the HBMYCNG gene, as well as chromosome  
20 sequences flanking that gene, that can be utilized as  
part of the methods of the present invention for the  
diagnosis or treatment of human disease; and (i) nucleic  
acid sequences that hybridize to the above-described  
sequences under stringent conditions. The nucleic acids  
25 of the invention include, but are not limited to, cDNA  
and genomic DNA sequences of the HBMYCNG gene.

The present invention also encompasses gene products  
of the nucleic acid molecules listed above; i.e.,  
proteins and/or polypeptides that are encoded by the  
30 above-disclosed HBMYCNG nucleic acid molecules and are  
expressed in recombinant host systems.

Antagonists and agonists of the HBMYCNG gene and/or  
gene product disclosed herein are also included in the  
present invention. Such antagonists and agonists will  
35 include, for example, small molecules, large molecules,  
and antibodies directed against the HBMYCNG gene product.  
Antagonists and agonists of the invention also include



nucleotide sequences, such as antisense and ribozyme  
5 molecules, and gene or regulatory sequence replacement  
constructs, that can be used to inhibit or enhance  
expression of the disclosed HBMYCNG nucleic acid  
molecules.

The present invention further encompasses cloning  
10 vectors, including expression vectors, that contain the  
nucleic acid molecules of the invention and can be used  
to express those nucleic acid molecules in host  
organisms. The present invention also relates to host  
cells engineered to contain and/or express the nucleic  
15 acid molecules of the invention. Further, host organisms  
that have been transformed with these nucleic acid  
molecules are also encompassed in the present invention,  
e.g., transgenic animals, particularly transgenic  
non-human animals, and more particularly transgenic  
20 non-human mammals.

The present invention also relates to methods and  
compositions for the diagnosis of human disease involving  
cation, e.g.,  $\text{Ca}^{2+}$ , sodium or potassium channel,  
dysfunction or lack of other ionic homeostasis including  
25 but not limited to, CNS disorders such as stroke, anxiety  
and depression, and degenerative neurological diseases,  
e.g., Alzheimer's disease or Parkinson's disease, or  
disorders such as cardiac disorders, e.g., arrhythmia,  
diabetes, chronic pain or other disorders such as  
30 hypercalcemia, hypercalciuria, or  $\text{Ca}^{2+}$ , sodium or  
potassium channel dysfunction that is associated with  
immunological disorders (GI) tract disorders, or renal or  
liver disease. The present invention further relates to  
methods and compositions useful for the diagnosis and  
35 treatment of diseases and conditions related to or  
involving the serotonin nervous system which participates  
in the control of anxiety, fear, depression, sleep and

pain. Accordingly, the present invention still further  
5 relates to methods and compositions for the diagnosis of anxiety and fear disorders, bipolar and major depression, panic disorder, headaches, migraine, disorders of circadian rhythmicity, stress, various sexual dysfunctions including but not limited to erectile  
10 dysfunction, neuroleptic-induced catalepsy, Rett syndrome and aggressive behaviors.

Such methods comprise, for example, measuring expression of the HBMYCNG gene in a patient sample, or detecting a mutation in the gene in the genome of a  
15 mammal, including a human, suspected of exhibiting ion channel dysfunction. The nucleic acid molecules of the invention can also be used as diagnostic hybridization probes or as primers for diagnostic PCR analysis to identify HBMYCNG gene mutations, allelic variations, or  
20 regulatory defects, such as defects in the expression of the gene. Such diagnostic PCR analyses can be used to diagnose individuals with disorders associated with a particular HBMYCNG gene mutation, allelic variation, or regulatory defect. Such diagnostic PCR analyses can also  
25 be used to identify individuals susceptible to ion channel disorders.

Methods and compositions, including pharmaceutical compositions, for the treatment of ion channel disorders are also included in the invention. Such methods and  
30 compositions are capable of modulating the level of HBMYCNG gene expression and/or the level of activity of the respective gene product. Such methods include, for example, modulating the expression of the HBMYCNG gene and/or the activity of the HBMYCNG gene product for the  
35 treatment of a disorder that is mediated by a defect in some other gene.

Such methods also include screening methods for the  
5 identification of compounds that modulate the expression  
of the nucleic acids and/or the activity of the  
polypeptides of the invention, e.g., assays that measure  
HBMYCNG mRNA and/or gene product levels, and assays that  
measure levels of HBMYCNG activity, such as the ability  
10 of the gene products to allow  $\text{Ca}^{2+}$  influx into cells.

For example, cellular and non-cellular assays are  
known that can be used to identify compounds that  
interact with the HBMYCNG gene and/or gene product, e.g.,  
modulate the activity of the gene and/or bind to the gene  
15 product. Such cell-based assays of the invention utilize  
cells, cell lines, or engineered cells or cell lines that  
express the gene product.

In one embodiment, such methods comprise contacting  
a compound to a cell that expresses the HBMYCNG gene,  
20 measuring the level of gene expression, gene product  
expression, or gene product activity, and comparing this  
level to the level of the HBMYCNG gene expression, gene  
product expression, or gene product activity produced by  
the cell in the absence of the compound, such that if the  
25 level obtained in the presence of the compound differs  
from that obtained in its absence, a compound that  
modulates the expression of the HBMYCNG gene and/or the  
synthesis or activity of the gene product has been  
identified.

In an alternative embodiment, such methods comprise  
30 administering a compound to a host organism, e.g., a  
transgenic animal that expresses a HBMYCNG transgene or a  
mutant HBMYCNG transgene, and measuring the level of  
HBMYCNG gene expression, gene product expression, or gene  
35 product activity. The measured level is compared to the  
level of HBMYCNG gene expression, gene product  
expression, or gene product activity in a host that is

- not exposed to the compound, such that if the level  
5 obtained when the host is exposed to the compound differs  
from that obtained when the host is not exposed to the  
compound, a compound that modulates the expression of the  
HBMYCNG gene and/or the synthesis or activity of HBMYCNG  
gene products has been identified.
- 10 The compounds identified by these methods include  
therapeutic compounds that can be used as pharmaceutical  
compositions to reduce or eliminate the symptoms of ion  
channel disorders such as CNS disorders, e.g., stroke,  
chronic pain, anxiety and depression, or degenerative  
15 neurological diseases such as Alzheimer's disease or  
Parkinson's disease, cardiac diseases or other  
ion-related disorders such as hypercalcemia,  
hypocalcemia, hypercalciuria, hypocalciuria, or ion  
disorders that are associated with immunological  
20 disorders, gastro-intestinal (GI) tract disorders, or  
renal or liver disease. Compounds identified by these  
methods further include compound useful for the treatment  
of diseases and conditions related to or involving the  
serotonin nervous system which participates in the  
25 control of anxiety, fear, depression, sleep and pain.  
Accordingly, compounds identified by these methods can be  
used for the treatment of anxiety and fear disorders,  
bipolar and major depression, panic disorder, headaches,  
migraine, disorders of circadian rhythmicity, stress,  
30 various sexual dysfunctions including but not limited to  
erectile dysfunction, neuroleptic-induced catalepsy, Rett  
syndrome and aggressive behaviors.

In another embodiment, screening methods are used  
for the detection, isolation, and identification of  
35 compounds which modulate the level of intracellular  
cyclic nucleotides. In one example, cells expressing the  
human HBMYCNG gene and a second biochemical activity

involved in cyclic nucleotide synthesis or degradation,  
 5 including but not limited to a G-protein coupled  
 receptor, are contacted with a test compound and the  
 level of calcium, or other cation, influx is determined.  
 Evaluation of calcium, or other cation, influx in the  
 presence or absence of the test compound indicates  
 10 whether that compound is an agonist or antagonist of  
 cyclic nucleotide accumulation within the cell.

Similarly, in another embodiment, such an assay can  
 be used to detect, isolate, and characterize the cognate  
 ligand recognized by an "orphan" G-protein coupled  
 15 receptor. In this embodiment, the cell expressing both  
 the human HBMYCNG gene and the orphan G-protein coupled  
 receptor is contacted with compounds and/or mixtures of  
 compounds, and human HBMYCNG mediated calcium, or other  
 cation, influx is determined with and without the test  
 20 compounds. Presence of the cognate ligand for the  
 "orphan" receptor is indicated by the intracellular  
 synthesis of cAMP and/or cGMP mediated by the activated  
 G-protein coupled receptor, leading to activation of the  
 HBMYCNG cation channel and an increase in calcium, or  
 25 other cation, influx into the cell.

#### 4. DESCRIPTION OF THE FIGURES

FIG. 1. Nucleotide sequence (SEQ ID NO:1) and  
 amino acid sequence (SEQ ID NO:2) of the full length cDNA  
 30 for Human HBMYCNG. The ATG initiation codon for HBMYCNG  
 translation is found at nucleotides 20-22, and the TAA  
 termination codon is found at nucleotides 2012-2014.

FIG. 2. Conceptual translation of the open reading  
 frame of the cDNA sequence of Figure 1, providing the  
 35 amino acid sequence Human HBMYCNG (SEQ ID NO:2).

FIG. 3. Conceptual translation of nucleotide 20 to  
 2011 of the 2186-nucleotide (SEQ ID NO:2), full length

Human HBMYCNG cDNA with the six transmembrane segments in  
 5 bold and the ion pore underlined.

FIG. 4. Amino acid Sequence alignment of Human  
 HBMYCNG (SEQ ID NO:2) and related rabbit (rACNG; gi  
 433960), bovine (CNG2\_BOS; gi 227199), murine  
 (CNG2\_mouse; gi 6671780), and rat (CNG2\_RAT; gi 227120)  
 10 cyclic nucleotide gated channels. Blackened areas  
 represent identical amino acids and the gray highlighted  
 residues indicate similar amino acids.

FIG. 5. Nucleotide sequence (SEQ ID NO:23) and  
 amino acid sequence (SEQ ID NO:24) of a variant of the  
 15 full length cDNA for Human HBMYCNG. The ATG initiation  
 codon for the variant HBMYCNG translation is found at  
 nucleotides 20-22, and the TAA termination codon is found  
 at nucleotides 2012-2014.

FIG. 6. Conceptual translation of the open reading  
 20 frame of the cDNA sequence of Figure 5, providing the  
 amino acid sequence variant Human HBMYCNG (SEQ ID NO:24).

FIG. 7. Amino acid Sequence alignment of the Human  
 HBMYCNG (SEQ ID NO:2) with the Human HBMYCNG variant (SEQ  
 ID NO:24). Vertical bars ("|") represent identical amino  
 25 acids. The threonine to isoleucine amino acid change in  
 the Human HBMYCNG variant sequence at amino acid position  
 442 of SEQ ID NO:24 is noted.

## 5. DETAILED DESCRIPTION OF THE INVENTION

30 The present invention relates to the isolation and  
 identification of novel nucleic acid molecules and  
 proteins and polypeptides for the formation or function  
 of novel human ion channels. More specifically, the  
 invention relates to a novel HBMYCNG human gene which  
 35 encodes the corresponding HBMYCNG protein or biologically  
 active derivatives or fragments thereof, involved in the  
 formation or function of cation channels.

The HBMYCNG nucleic acid molecules of the present invention include isolated naturally-occurring or recombinantly-produced human HBMYCNG nucleic acid molecules, e.g., DNA molecules, cloned genes or degenerate variants thereof. The compositions of the invention also include isolated, naturally-occurring or recombinantly-produced human HBMYCNG protein or polypeptide.

Other embodiments of the invention include antibodies directed to the HBMYCNG protein or polypeptide of the invention and methods and compositions for the diagnosis and treatment of human diseases related to ion channel dysfunction as described below.

#### 5.1. The HBMYCNG Nucleic Acids of the Invention

The complete HBMYCNG gene of the invention, HBMYCNG, is a novel, complete human nucleic acid molecule that encodes a protein or polypeptide involved in the formation or function of a novel human ion channel. Although this gene and the protein encoded therein display sequence and structural homology to other cation channel proteins known in the art, it is also known in the art that proteins displaying these homologies have significant differences in function, such as conductance and permeability, as well as differences in tissue expression, as well as co-expression, or not, of different CNG  $\beta$ -subunit polypeptides. As such, it is acknowledged in the art that nucleic acid molecules and the proteins encoded by those molecules sharing these homologies can still represent diverse, distinct and unique nucleic acids and proteins, respectively.

The HBMYCNG nucleic acid molecules of the invention include the following: (a) a nucleic acid molecule comprising the DNA sequence, HBMYCNG, as shown in FIG. 1

- or FIG. 5; (b) any nucleic acid sequence that encodes the amino acid sequence, HBMYCNG as shown in FIG. 2 or FIG. 6; (c) any nucleic acid sequence that hybridizes to the complement of DNA sequences that encode the amino acid sequences of FIG. 2 or FIG.6 under highly stringent conditions, e.g., hybridization to filter-bound DNA in 0.5 M NaHPO<sub>4</sub>, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (see, e.g., Ausubel F.M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3) or (d) any nucleic acid sequence that hybridizes to the complement of DNA sequences that encode the amino acid sequences of FIG. 2 or FIG.6, under less stringent conditions, such as moderately stringent conditions, e.g., washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel et al., 1989, *supra*), and which encodes a gene product functionally equivalent to a HBMYCNG gene product encoded by the deposited sequences or the sequence depicted in FIG. 2 or FIG. 6. "Functionally equivalent" as used herein refers to any protein capable of exhibiting a substantially similar *in vivo* or *in vitro* activity as the HBMYCNG gene product encoded by the HBMYCNG nucleic acid molecules described herein, e.g., ion channel formation or function. For the purposes of the present invention, the HBMYCNG nucleic acid as depicted in FIG. 1 is functionally equivalent to the HBMYCNG nucleic acid as depicted in FIG. 5.

As used herein, the term "HBMYCNG nucleic acid molecule" may also refer to fragments and/or degenerate variants of DNA sequences (a) through (d), including naturally occurring variants or mutant alleles thereof. Such fragments include, for example, nucleotide sequences that encode portions of the HBMYCNG protein that



correspond to functional domains of the protein. One  
5 embodiment of such a HBMYCNG nucleic acid fragment  
comprises a nucleic acid that encodes the fifth and sixth  
transmembrane segments of the HBMYCNG protein, including  
the predicted pore loop (see FIG. 3).

Additionally, the HBMYCNG nucleic acid molecules of  
10 the invention include isolated nucleic acid molecules,  
preferably DNA molecules, that hybridize under highly  
stringent or moderately stringent hybridization  
conditions to at least about 6, preferably at least about  
12, and more preferably at least about 18, consecutive  
15 nucleotides of the nucleic acid sequences of (a) through  
(d), identified *supra*.

The HBMYCNG nucleic acid molecules of the invention  
also include nucleic acid molecules, preferably DNA  
molecules, that hybridize to, and are therefore  
20 complements of, the DNA sequences of (a) through (d),  
*supra*. Such hybridization conditions may be highly  
stringent or moderately stringent, as described above. In  
those instances in which the nucleic acid molecules are  
deoxyoligonucleotides ("oligos"), highly stringent  
25 conditions may include, e.g., washing in 6xSSC/0.05%  
sodium pyrophosphate at 37°C (for 14-base oligos), 48°C  
(for 17-base oligos), 55°C (for 20-base oligos), and 60°C  
(for 23-base oligos). These nucleic acid molecules may  
encode or act as HBMYCNG antisense molecules useful, for  
30 example, in HBMYCNG gene regulation or as antisense  
primers in amplification reactions of HBMYCNG nucleic  
acid sequences. Further, such sequences may be used as  
part of ribozyme and/or triple helix sequences, also  
useful for HBMYCNG gene regulation. Still further, such  
35 molecules may be used as components of diagnostic methods  
whereby, for example, the presence of a particular  
HBMYCNG allele or alternatively spliced HBMYCNG

transcript responsible for causing or predisposing one to  
5 a disorder involving ion channel dysfunction may be  
detected.

Typically, the HBMYCNG nucleic acids of the  
invention should exhibit at least about 90% overall  
homology at the nucleotide level, and more preferably at  
10 least about 95% overall homology to the nucleic acid  
sequence of FIG. 1.

Also included within the HBMYCNG nucleic acids of  
the invention are nucleic acid molecules, preferably DNA  
molecules, comprising an HBMYCNG nucleic acid, as  
15 described herein, operatively linked to a nucleotide  
sequence encoding a heterologous protein or peptide.

To determine the percent identity of two nucleic  
acid sequences or of two amino acid sequences, the  
sequences are aligned for optimal comparison purposes  
20 (e.g., gaps can be introduced in the sequence of a first  
amino acid or nucleic acid sequence for optimal alignment  
with a second amino acid or nucleic acid sequence). The  
amino acid residues or nucleotides at corresponding amino  
acid positions or nucleotide positions are then compared.  
25 When a position in the first sequence is occupied by the  
same amino acid residue or nucleotide as the  
corresponding position in the second sequence, then the  
molecules are identical at that position. The percent  
identity between the two sequences is a function of the  
30 number of identical positions shared by the sequences  
(i.e., % identity = number of identical overlapping  
positions/total number of positions x 100%). In one  
embodiment, the two sequences are the same length.

The determination of percent identity between two  
35 sequences can also be accomplished using a mathematical  
algorithm. A preferred, non-limiting example of a  
mathematical algorithm utilized for the comparison of two

- sequences is the algorithm of Karlin and Altschul, 1990,  
5 Proc. Natl. Acad. Sci. U.S.A. 87:2264-2268, modified as  
in Karlin and Altschul, 1993, Proc. Natl. Acad. Sci.  
U.S.A. 90: 5873-5877. Such an algorithm is incorporated  
into the NBLAST and XBLAST programs of Altschul et al.,  
1990, J. Mol. Biol. 215: 403. BLAST nucleic acid searches  
10 can be performed with the NBLAST nucleic acid program  
parameters set, e.g., for score=100, wordlength=12 to  
obtain nucleic acid sequences homologous to a nucleic  
acid molecule of the present invention. BLAST polypeptide  
searches can be performed with the XBLAST program  
15 parameters set, e.g., to score=50, wordlength=3 to obtain  
amino acid sequences homologous to a polypeptide molecule  
of the present invention. To obtain gapped alignments for  
comparison purposes, Gapped BLAST can be utilized as  
described in Altschul et al., 1997, Nucleic Acids Res.  
20 25: 3389-3402. Alternatively, PSI-BLAST can be used to  
perform an iterated search which detects distant  
relationships between molecules (Id.). When utilizing  
BLAST, Gapped BLAST, and PSI-Blast programs, the default  
parameters of the respective programs (e.g., of XBLAST  
25 and NBLAST) can be used (e.g.,  
<http://www.ncbi.nlm.nih.gov>). Another preferred,  
non-limiting example of a mathematical algorithm utilized  
for the comparison of sequences is the algorithm of Myers  
and Miller, 1988, CABIOS 4:11-17. Such an algorithm is  
30 incorporated in the ALIGN program (version 2.0) which is  
part of the GCG sequence alignment software package. When  
utilizing the ALIGN program for comparing amino acid  
sequences, a PAM120 weight residue table, a gap length  
penalty of 12, and a gap penalty of 4 can be used. Still  
35 another preferred algorithm for the comparison of  
polypeptide sequences is that of Thompson et al.,

designated CLUSTALW, which is disclosed in Thompson et  
5 al. 1994 Nucleic Acids Research 2(22): 4673-80.

The percent identity between two sequences can be  
determined using techniques similar to those described  
above, with or without allowing gaps. In calculating  
percent identity, typically only exact matches are  
10 counted.

Moreover, due to the degeneracy of the genetic code,  
other DNA sequences that encode substantially the amino  
acid sequence of HBMYCNG may be used in the practice of  
the present invention for the cloning and expression of  
15 HBMYCNG polypeptides. Such DNA sequences include those  
that are capable of hybridizing to the HBMYCNG nucleic  
acids of this invention under stringent (high or  
moderate) conditions, or that would be capable of  
hybridizing under stringent conditions but for the  
20 degeneracy of the genetic code.

Altered HBMYCNG DNA sequences that may be used in  
accordance with the invention include deletions,  
additions or substitutions of different nucleotide  
residues resulting in a nucleic acid molecule that  
25 encodes the same or a functionally equivalent gene  
product as those described *supra*. The gene product itself  
may contain deletions, additions or substitutions of  
amino acid residues within the HBMYCNG protein sequence,  
which result in a silent change, thus producing a  
30 functionally equivalent HBMYCNG polypeptide. Such amino  
acid substitutions may be made on the basis of similarity  
in polarity, charge, solubility, hydrophobicity,  
hydrophilicity, and/or the amphipatic nature of the  
residues involved. For example, negatively-charged amino  
35 acids include aspartic acid and glutamic acid;  
positively-charged amino acids include lysine and  
arginine; amino acids with uncharged polar head groups

having similar hydrophilicity values include the  
5 following: leucine, isoleucine, valine; glycine, aniline;  
asparagine, glutamine; serine, threonine; phenylalanine,  
tyrosine. A functionally equivalent HBMYCNG polypeptide  
can include a polypeptide which displays the same type of  
biological activity (e.g., cation channel) as the native  
10 HBMYCNG protein, but not necessarily to the same extent.

The nucleic acid molecules or sequences of the  
invention may be engineered in order to alter the HBMYCNG  
coding sequence for a variety of ends including but not  
limited to alterations that modify processing and  
15 expression of the gene product. For example, mutations  
may be introduced using techniques which are well known  
in the art, e.g., site-directed mutagenesis, to insert  
new restriction sites, to alter glycosylation patterns,  
phosphorylation, etc. For example, in certain expression  
20 systems such as yeast, host cells may over-glycosylate  
the gene product. When using such expression systems, it  
may be preferable to alter the HBMYCNG coding sequence to  
eliminate any N-linked glycosylation sites.

In another embodiment of the invention, the HBMYCNG  
25 nucleic acid or a modified HBMYCNG sequence may be  
ligated to a heterologous sequence to encode a fusion  
protein. The fusion protein may be engineered to contain  
a cleavage site located between the HBMYCNG sequence and  
the heterologous protein sequence, so that the HBMYCNG  
30 protein can be cleaved away from the heterologous moiety.

The HBMYCNG nucleic acid molecules of the invention  
can also be used as hybridization probes for obtaining  
HBMYCNG cDNAs or genomic HBMYCNG DNA. In addition, the  
nucleic acids of the invention can be used as primers in  
35 PCR amplification methods to isolate HBMYCNG cDNAs and  
genomic DNA, e.g., from other species.

The HBMYCNG gene sequences of the invention may also  
5 used to isolate mutant HBMYCNG gene alleles. Such mutant  
alleles may be isolated from individuals either known or  
proposed to have a genotype related to ion channel  
dysfunction. Mutant alleles and mutant allele gene  
products may then be utilized in the screening,  
10 therapeutic and diagnostic systems described in Section  
5.4., *infra*. Additionally, such HBMYCNG gene sequences  
can be used to detect HBMYCNG gene regulatory (e.g.,  
promoter) defects which can affect ion channel function.

A cDNA of a mutant HBMYCNG gene may be isolated, for  
15 example, by using PCR, a technique which is well known to  
those of skill in the art (see, e.g., U.S. Patent No.  
4,683,202). The first cDNA strand may be synthesized by  
hybridizing an oligo-dT oligonucleotide to mRNA isolated  
from tissue known or suspected to be expressed in an  
20 individual putatively carrying the mutant HBMYCNG allele,  
and by extending the new strand with reverse  
transcriptase. The second strand of the cDNA is then  
synthesized using an oligonucleotide that hybridizes  
specifically to the 5' end of the normal gene. Using  
25 these two primers, the product is then amplified via PCR,  
cloned into a suitable vector, and subjected to DNA  
sequence analysis through methods well known in the art.  
By comparing the DNA sequence of the mutant HBMYCNG  
allele to that of the normal HBMYCNG allele, the  
30 mutation(s) responsible for the loss or alteration of  
function of the mutant HBMYCNG gene product can be  
ascertained.

Alternatively, a genomic library can be constructed  
using DNA obtained from an individual suspected of or  
35 known to carry the mutant HBMYCNG allele, or a cDNA  
library can be constructed using RNA from a tissue known,  
or suspected, to express the mutant HBMYCNG allele. The

normal HBMYCNG gene or any suitable fragment thereof may  
5 then be labeled and used as a probe to identify the  
corresponding mutant HBMYCNG allele in such libraries.  
Clones containing the mutant HBMYCNG gene sequences may  
then be purified and subjected to sequence analysis  
according to methods well known in the art.

10 According to another embodiment, an expression  
library can be constructed utilizing cDNA synthesized  
from, for example, RNA isolated from a tissue known, or  
suspected, to express a mutant HBMYCNG allele in an  
individual suspected of or known to carry such a mutant  
15 allele. Gene products made by the putatively mutant  
tissue may be expressed and screened using standard  
antibody screening techniques in conjunction with  
antibodies raised against the normal HBMYCNG gene  
product, as described in Section 5.3, *supra*. For  
20 screening techniques, see, for example, Harlow, E. and  
Lane, eds., 1988, "Anti-bodies: A Laboratory Manual",  
Cold Spring Harbor Press, Cold Spring Harbor.

In cases where a HBMYCNG mutation results in an  
expressed gene product with altered function (e.g., as a  
25 result of a missense or a frameshift mutation), a  
polyclonal set of anti-HBMYCNG gene product antibodies  
are likely to cross-react with the mutant HBMYCNG gene  
product. Library clones detected via their reaction with  
such labeled antibodies can be purified and subjected to  
30 sequence analysis according to methods well known to  
those of skill in the art.

In an alternate embodiment of the invention, the  
coding sequence of HBMYCNG can be synthesized in whole or  
in part, using chemical methods well known in the art,  
35 based on the nucleic acid and/or amino acid sequences of  
the HBMYCNG genes and proteins disclosed herein. See, for  
example, Caruthers et al., 1980, Nuc. Acids Res. Symp.

- Ser. 7: 215-233; Crea and Horn, 1980, Nuc. Acids Res.
- 5 9(10): 2331; Matteucci and Caruthers, 1980, Tetrahedron Letters 21: 719; and Chow and Kempe, 1981, Nuc. Acids Res. 9(12): 2807-2817.

The invention also encompasses (a) DNA vectors that contain any of the foregoing HBMYCNG sequences and/or

10 their complements; (b) DNA expression vectors that contain any of the foregoing HBMYCNG coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences; and (c) genetically engineered host cells that contain any of the

15 foregoing HBMYCNG coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences in the host cell. As used herein, regulatory elements include, but are not limited to inducible and non-inducible promoters, enhancers,

20 operators and other elements known to those skilled in the art that drive and regulate expression. Such regulatory elements include but are not limited to the cytomegalovirus hCMV immediate early gene, the early or late promoters of SV40 adenovirus, the *lac* system, the

25 *trp* system, the *TAC* system, the *TRC* system, the major operator and promoter regions of phage A, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase, the promoters of acid phosphatase, and the promoters of the yeast  $\alpha$ -mating

30 factors.

The invention still further includes nucleic acid analogs, including but not limited to peptide nucleic acid analogues, equivalent to the nucleic acid molecules described herein. "Equivalent" as used in this context

35 refers to nucleic acid analogs that have the same primary base sequence as the nucleic acid molecules described above. Nucleic acid analogs and methods for the synthesis



of nucleic acid analogs are well known to those of skill  
 5 in the art. See, e.g., Egholm, M. et al., 1993, Nature  
 365:566-568; and Perry-O'Keefe, H. et al., 1996, Proc.  
 Natl. Acad. USA 93:14670-14675.

## 5.2. HBMYCNG Proteins and Polypeptides of the Invention

10 The HBMYCNG nucleic acid molecules of the invention  
 may be used to generate recombinant DNA molecules that  
 direct the expression in appropriate host cells of  
 HBMYCNG polypeptides, including the full-length HBMYCNG  
 protein, functionally active or equivalent HBMYCNG  
 15 proteins and polypeptides, e.g., mutated, truncated or  
 deleted forms of HBMYCNG, peptide fragments of HBMYCNG,  
 or HBMYCNG fusion proteins. A functionally equivalent  
 HBMYCNG polypeptide can include a polypeptide which  
 displays the same type of biological activity (e.g.,  
 20 cation channel formation and/or function) as the native  
 HBMYCNG protein, but not necessarily to the same extent.

In a preferred embodiment, the proteins and  
 polypeptides of the invention include the HBMYCNG amino  
 acid sequence depicted in FIG. 2, which corresponds to  
 25 the conceptual translation of the nucleotide sequence  
 spanning residues 20 to 2011 of the cDNA sequence of  
 HBMYCNG, as depicted in FIG. 1. This amino acid sequence  
 includes six transmembrane domains and an overall  
 topology that is conserved in CNG ion channels.

30 In other embodiments of the present invention the  
 proteins and polypeptides of the invention include the  
 HBMYCNG amino acid sequence depicted in FIG. 2 except for  
 the initial methionine residue; *i.e.*, a polypeptide  
 having an amino acid sequence corresponding to amino  
 35 acids 2 through 664 the amino acid sequence of FIG. 2,  
 which corresponds to the conceptual translation of the

nucleotide sequence spanning residues 23 to 2011 of the  
 5 cDNA sequence of HBMYCNG, as depicted in FIG. 1.

The HBMYCNG amino acid sequence of FIG. 2, which has  
 a calculated molecular weight of 75.9 kDa, is homologous  
 to four cyclic nucleotide gated proteins. A comparison of  
 the HBMYCNG amino acid sequence with that of rabbit  
 10 (rACNG; gi 433960), bovine (CNG2\_BOS; gi 227199), mouse  
 (CNG2\_mouse; gi 6671780), and rat (CNG2\_RAT; gi 227120)  
 cyclic nucleotide gated channels from rabbit is presented  
 in FIG. 4. The amino acid sequences for Human HBMYCNG and  
 for rabbit aorta rCNG displayed 95.633% similarity and  
 15 93.675% identity; the amino acid sequences for Human  
 HBMYCNG and for bovine olfactory CNG2\_BOVIN displayed  
 95.324% similarity and 93.213% identity; the amino acid  
 sequences for Human HBMYCNG and for murine olfactory  
 CNG2\_MOUSE displayed 94.260% similarity and 93.051%  
 20 identity; and the amino acid sequences for Human HBMYCNG  
 and for rat olfactory CNG2\_RAT displayed 94.109%  
 similarity and 92.598% identity.

The HBMYCNG proteins and polypeptides of the  
 invention include peptide fragments of HBMYCNG, e.g.,  
 25 peptides corresponding to one or more domains of the  
 protein, mutated, truncated or deleted forms of the  
 proteins and polypeptides, as well as HBMYCNG fusion  
 proteins, all of which derivatives of HBMYCNG can be  
 obtained by techniques well known in the art, given the  
 30 HBMYCNG nucleic acid and amino acid sequences disclosed  
 herein.

As noted in Section 5.1, *supra*, the proteins and  
 polypeptides of the invention may contain deletions,  
 additions or substitutions of amino acid residues within  
 35 the HBMYCNG protein sequence, which result in a silent  
 change, thus producing a functionally equivalent HBMYCNG  
 polypeptide. Such amino acid substitutions may be made on

the basis of similarity in polarity, charge, solubility,  
5 hydrophobicity, hydrophilicity, and/or the amphipatic  
nature of the residues involved. For example,  
negatively-charged amino acids include aspartic acid and  
glutamic acid; positively-charged amino acids include  
lysine, arginine and histidine; amino acids with  
10 uncharged polar head groups having similar hydrophilicity  
values include the following: leucine, isoleucine,  
valine, glycine, alanine, asparagine, glutamine, serine,  
threonine, phenylalanine, tyrosine.

Mutated or altered forms of the HBMYCNG proteins and  
15 polypeptides of the invention can be obtained using  
either random mutagenesis techniques or site-directed  
mutagenesis techniques well known in the art or by  
chemical methods, e.g., protein synthesis techniques (see  
Section 5.1, *supra*). Mutant HBMYCNG proteins or  
20 polypeptides can be engineered so that regions important  
for function are maintained, while variable residues are  
altered, e.g., by deletion or insertion of an amino acid  
residue(s) or by the substitution of one or more  
different amino acid residues. For example, conservative  
25 alterations at the variable positions of a polypeptide  
can be engineered to produce a mutant HBMYCNG polypeptide  
that retains the function of HBMYCNG. Non-conservative  
alterations of variable regions can be engineered to  
alter HBMYCNG function, if desired. Alternatively, in  
30 those cases where modification of function (either to  
increase or decrease function) is desired, deletion or  
non-conservative alterations of conserved regions of the  
polypeptide may be engineered.

Fusion proteins containing HBMYCNG amino acid  
35 sequences can also be obtained by techniques known in the  
art, including genetic engineering and chemical protein  
synthesis techniques. According to a preferred

embodiment, the fusion proteins of the invention are  
 5 encoded by an isolated nucleic acid molecule comprising  
 an HBMYCNG nucleic acid of the invention that encodes a  
 polypeptide with an activity of a HBMYCNG protein, or a  
 fragment thereof, linked in frame and uninterrupted by  
 stop codons to a nucleotide sequence that encodes a  
 10 heterologous protein or peptide.

The fusion proteins of the invention include those  
 that contain the full length HBMYCNG amino acid sequence,  
 an HBMYCNG peptide sequence, e.g., encoding one or more  
 functional domains, a mutant HBMYCNG amino acid sequence  
 15 or a truncated HBMYCNG amino acid sequence linked to an  
 unrelated protein or polypeptide sequence. Such fusion  
 proteins include but are not limited to IgFc fusions  
 which stabilize the HBMYCNG fusion protein and may  
 prolong half-life of the protein *in vivo* or fusions to an  
 20 enzyme, fluorescent protein or luminescent protein that  
 provides a marker function.

According to a preferred embodiment, the HBMYCNG  
 nucleic acid molecules of the invention may be used to  
 generate recombinant DNA molecules that direct the  
 25 expression of HBMYCNG polypeptides, including the  
 full-length HBMYCNG protein, e.g., HBMYCNG or  
 functionally active or equivalent HBMYCNG peptides  
 thereof, or HBMYCNG fusion proteins in appropriate host  
 cells.

30 In order to express a biologically active HBMYCNG  
 polypeptide, a nucleic acid molecule coding for the  
 polypeptide, or a functional equivalent thereof as  
 described in Section 5.1, *supra*, is inserted into an  
 appropriate expression vector, i.e., a vector which  
 35 contains the necessary elements for the transcription and  
 translation of the inserted coding sequence. The HBMYCNG  
 gene products so produced, as well as host cells or cell

lines transfected or transformed with recombinant HBMYCNG  
 5 expression vectors, can be used for a variety of  
 purposes. These include but are not limited to generating  
 antibodies (i.e., monoclonal or polyclonal) that bind to  
 the HBMYCNG protein, including those that competitively  
 inhibit binding and thus can "neutralize" HBMYCNG  
 10 activity, and the screening and selection of HBMYCNG  
 analogs or ligands.

Methods which are well known to those skilled in the  
 art are used to construct expression vectors containing  
 the HBMYCNG coding sequences of the invention and  
 15 appropriate transcriptional and translational control  
 signals. These methods include *in vitro* recombinant DNA  
 techniques, synthetic techniques and *in vivo*  
 recombination/genetic recombination. See, for example,  
 the techniques described in Maniatis et al., 1989,  
 20 Molecular Cloning, A Laboratory Manual, Cold Spring  
 Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current  
 Protocols in Molecular Biology, Greene Publishing  
 Associates and Wiley Interscience, N.Y. See also Sambrook  
 et al., 1989, Molecular Cloning, A Laboratory Manual,  
 25 Cold Spring Harbor Press, N.Y.

A variety of host-expression vector systems may be  
 used to express the HBMYCNG coding sequences of this  
 invention. Such host-expression systems represent  
 vehicles by which the coding sequences of interest may be  
 30 produced and subsequently purified, but also represent  
 cells which may, when transformed or transfected with the  
 appropriate nucleotide coding sequences, exhibit the  
 corresponding HBMYCNG gene products *in situ* and/or  
 function *in vivo*. These hosts include but are not limited  
 35 to microorganisms such as bacteria (e.g., *E. coli*, *B.*  
*subtilis*) transformed with recombinant bacteriophage DNA,  
 plasmid DNA or cosmid DNA expression vectors containing

the HBMYCNG coding sequences; yeast (e.g., *Saccharomyces*,  
 5 *Pichia*) transformed with recombinant yeast expression  
 vectors containing the HBMYCNG coding sequence; insect  
 cell systems infected with recombinant virus expression  
 vectors (e.g., *baculovirus*) containing the HBMYCNG coding  
 sequence; plant cell systems infected with recombinant  
 10 virus expression vectors (e.g., cauliflower mosaic virus,  
 CaMV; tobacco mosaic virus, TMV) or transformed with  
 recombinant plasmid expression vectors (e.g., Ti plasmid)  
 containing the HBMYCNG coding sequence; or mammalian cell  
 systems (e.g., COS, CHO, BHK, 293, 3T3) harboring  
 15 recombinant expression constructs containing promoters  
 derived from the genome of mammalian cells (e.g., the  
 metallothionein promoter) or from mammalian viruses  
 (e.g., the adenovirus late promoter or vaccinia virus  
 7.5K promoter).

20 The expression elements of these systems can vary in  
 their strength and specificities. Depending on the  
 host/vector system utilized, any of a number of suitable  
 transcription and translation elements, including  
 constitutive and inducible promoters, may be used in the  
 25 expression vector. For example, when cloning in bacterial  
 systems, inducible promoters such as pL of bacteriophage  
 $\phi$ , plac, ptrp, ptac (ptrp-lac hybrid promoter) and the  
 like may be used; when cloning in insect cell systems,  
 promoters such as the baculovirus polyhedrin promoter may  
 30 be used; when cloning in plant cell systems, promoters  
 derived from the genome of plant cells (e.g., heat shock  
 promoters; the promoter for the small subunit of RUBISCO;  
 the promoter for the chlorophyll a/b binding protein) or  
 from plant viruses (e.g., the 35S RNA promoter of CaMV;  
 35 the coat protein promoter of TMV) may be used; when  
 cloning in mammalian cell systems, promoters derived from  
 the genome of mammalian cells (e.g., metallothionein

promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used; when generating cell lines that contain multiple copies of the HBMYCNG DNA, SV40-, BPV- and EBV-based vectors may be used with an appropriate selectable marker.

10 In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the HBMYCNG expressed. For example, when large quantities of an HBMYCNG polypeptide are to be produced, e.g., for the generation of antibodies or the  
 15 production of the HBMYCNG gene product, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include but are not limited to the *E. coli* expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:  
 20 1791), in which the HBMYCNG coding sequence may be ligated into the vector in frame with the lacZ coding region so that a hybrid HBMYCNG/lacZ protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res.  
 13: 3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem.  
 25 264: 5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by affinity chromatography, e.g.,  
 30 adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety. See also  
 35 Booth et al., 1988, Immunol. Lett. 19: 65-70; and Gardella et al., 1990, J. Biol. Chem. 265: 15854-15859; Pritchett et al., 1989, Biotechniques 7: 580.

In yeast, a number of vectors containing

5 constitutive or inducible promoters may be used. For a review, see Current Protocols in Molecular Biology, Vol. 2, 1988, Ed. Ausubel et al., Greene Publish. Assoc. & Wiley Interscience, Ch. 13; Grant et al., 1987, Expression and Secretion Vectors for Yeast, in Methods in

10 Enzymology, Eds. Wu & Grossman, 1987, Acad. Press, N.Y., Vol. 153, pp. 516-544; Glover, 1986, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3; and Bitter, 1987, Heterologous Gene Expression in Yeast, Methods in Enzymology, Eds. Berger & Kimmel, Acad. Press, N.Y., Vol.

15 152, pp. 673-684; and The Molecular Biology of the Yeast *Saccharomyces*, 1982, Cold Spring Harbor Press, Vols. I and II.

In an insect system, *Autographa californica* nuclear polyhydrosis virus (AcNPV) can be used as a vector to

20 express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The HBMYCNG coding sequence may be cloned into non-essential regions (for example, the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example, the polyhedrin promoter).

25 Successful insertion of the HBMYCNG coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses can then be

30 used to infect *Spodoptera frugiperda* cells in which the inserted gene is expressed (see e.g., Smith et al., 1983, J. Virol. 46: 584; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an

35 adenovirus is used as an expression vector, the HBMYCNG coding sequence may be ligated to an adenovirus transcription/translation control complex, e.g., the late



promoter and tripartite leader sequence. This chimeric  
 5 gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing HBMYCNG in infected hosts (see,  
 10 e.g., Logan & Shenk, 1984, Proc. Natl. Acad. Sci. (USA) 81: 3655-3659). Alternatively, the vaccinia 7.5K promoter may be used (see, e.g., Mackett et al., 1982, Proc. Natl. Acad. Sci. (USA) 79: 7415-7419; Mackett et al., 1984, J. Virol. 49: 857-864; Panicali et al., 1982, Proc. Natl.  
 15 Acad. Sci. 79: 4927-4931).

Specific initiation signals may also be required for efficient translation of inserted HBMYCNG coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where the entire HBMYCNG  
 20 gene, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the HBMYCNG coding sequence is inserted, exogenous  
 25 translational control signals, including the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the HBMYCNG coding sequence to ensure translation of the entire insert. These exogenous translational control  
 30 signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see, e.g., Bittner et  
 35 al., 1987, Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or

modifies and processes the gene product in the specific  
5 fashion desired. Such modifications (e.g., glycosylation)  
and processing (e.g., cleavage) of protein products may  
be important for the function of the protein. Different  
host cells have characteristic and specific mechanisms  
for the post-translational processing and modification of  
10 proteins. Appropriate cells lines or host systems can be  
chosen to ensure the correct modification and processing  
of the foreign protein expressed. To this end, eukaryotic  
host cells which possess the cellular machinery for  
proper processing of the primary transcript,  
15 glycosylation, and phosphorylation of the gene product  
may be used. Such mammalian host cells include but are  
not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293,  
WI38, etc.

For long-term, high-yield production of recombinant  
20 proteins, stable expression is preferred. For example,  
cell lines which stably express the HBMYCNG polypeptides  
of this invention may be engineered. Thus, rather than  
using expression vectors which contain viral origins of  
replication, host cells can be transformed with HBMYCNG  
25 nucleic acid molecules, e.g., DNA, controlled by  
appropriate expression control elements (e.g., promoter,  
enhancer, sequences, transcription terminators,  
polyadenylation sites, etc.), and a selectable marker.  
Following the introduction of foreign DNA, engineered  
30 cells may be allowed to grow for 1-2 days in an enriched  
media, and then are switched to a selective media. The  
selectable marker in the recombinant plasmid confers  
resistance to the selection and allows cells to stably  
integrate the plasmid into their chromosomes and grow to  
35 form foci which in turn can be cloned and expanded into  
cell lines. This method may advantageously be used to  
engineer cell lines which express HBMYCNG polypeptides on

the cell surface. Such engineered cell lines are particularly useful in screening for HBMYCNG analogs or ligands.

In instances where the mammalian cell is a human cell, among the expression systems by which the HBMYCNG nucleic acid sequences of the invention can be expressed are human artificial chromosome (HAC) systems (see, e.g., Harrington et al., 1997, *Nature Genetics* 15: 345-355).

HBMYCNG gene products can also be expressed in transgenic animals such as mice, rats, rabbits, guinea pigs, pigs, micro-pigs, sheep, goats, and non-human primates, e.g., baboons, monkeys, and chimpanzees. The term "transgenic" as used herein refers to animals expressing HBMYCNG nucleic acid sequences from a different species (e.g., mice expressing human HBMYCNG nucleic acid sequences), as well as animals that have been genetically engineered to overexpress endogenous (i.e., same species) HBMYCNG nucleic acid sequences or animals that have been genetically engineered to no longer express endogenous HBMYCNG nucleic acid sequences (i.e., "knock-out" animals), and their progeny.

Transgenic animals according to this invention may be produced using techniques well known in the art, including but not limited to pronuclear microinjection (Hoppe, P.C. and Wagner, T.E., 1989, U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten et al., 1985, *Proc. Natl. Acad. Sci., USA* 82: 6148-6152); gene targeting in embryonic stem cells (Thompson et al., 1989, *Cell* 56: 313-321); electroporation of embryos (Lo, 1983, *Mol Cell. Biol.* 3: 1803-1814); and sperm-mediated gene transfer (Lavitrano et al., 1989, *Cell* 57: 717-723); etc. For a review of such techniques, see Gordon, 1989, *Transgenic Animals*, *Intl. Rev. Cytol.* 115: 171-229.

In addition, any technique known in the art may be  
5 used to produce transgenic animal clones containing a  
HBMYCNG transgene, for example, nuclear transfer into  
enucleated oocytes of nuclei from cultured embryonic,  
fetal or adult cells induced to quiescence (Campbell et  
al., 1996, Nature 380: 64-66; Wilmut et al., 1997, Nature  
10 385: 810-813).

Host cells which contain the HBMYCNG coding sequence  
and which express a biologically active gene product may  
be identified by at least four general approaches; (a)  
DNA-DNA or DNA-RNA hybridization; (b) the presence or  
15 absence of "marker" gene functions; (c) assessing the  
level of transcription as measured by the expression of  
HBMYCNG mRNA transcripts in the host cell; and (d)  
detection of the gene product as measured by immunoassay  
or by its biological activity.

20 In the first approach, the presence of the HBMYCNG  
coding sequence inserted in the expression vector can be  
detected by DNA-DNA or DNA-RNA hybridization using probes  
comprising nucleotide sequences that are homologous to  
the HBMYCNG coding sequence, respectively, or portions or  
25 derivatives thereof.

In the second approach, the recombinant expression  
vector/host system can be identified and selected based  
upon the presence or absence of certain "marker" gene  
functions. For example, if the HBMYCNG coding sequence is  
30 inserted within a marker gene sequence of the vector,  
recombinants containing the HBMYCNG coding sequence can  
be identified by the absence of the marker gene function.  
Alternatively, a marker gene can be placed in tandem with  
the HBMYCNG sequence under the control of the same or  
35 different promoter used to control the expression of the  
HBMYCNG coding sequence. Expression of the marker in

response to induction or selection indicates expression  
5 of the HBMYCNG coding sequence.

Selectable markers include resistance to  
antibiotics, resistance to methotrexate, transformation  
phenotype, and occlusion body formation in baculovirus.  
In addition, thymidine kinase activity (Wigler et al.,  
10 1977, Cell 11: 223) hypoxanthine-guanine  
phosphoribosyltransferase (Szybalska & Szybalski, 1962,  
Proc. Natl. Acad. Sci. USA 48: 2026), and adenine  
phosphoribosyltransferase (Lowy et al., 1980, Cell 22:  
817) genes can be employed in tk-, hgprt- or apt- cells,  
15 respectively. Also, antimetabolite resistance can be used  
as the basis of selection for dhfr, which confers  
resistance to methotrexate (Wigler et al., 1980, Proc.  
Natl. Acad. Sci. USA 77: 3567; O'Hare et al., 1981, Proc.  
Natl. Acad. Sci. USA 78: 1527); gpt, which confers  
20 resistance to mycophenolic acid (Mulligan & Berg, 1981,  
Proc. Natl. Acad. Sci. USA 78: 2072); neo, which confers  
resistance to the aminoglycoside G-418 (Colberre-Garapin,  
et al., 1981, J. Mol. Biol. 150: 1); and hygromycin (Santerre et al., 1984,  
25 Gene 30: 147). Additional selectable genes have been  
described, namely trpB, which allows cells to utilize  
indole in place of tryptophan; hisD, which allows cells  
to utilize histinol in place of histidine (Hartman &  
Mulligan, 1988, Proc. Natl. Acad. Sci. USA 85: 8047); and  
30 ODC (ornithine decarboxylase) which confers resistance to  
the ornithine decarboxylase inhibitor,  
2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, 1987,  
in Current Communications in Molecular Biology, Cold  
Spring Harbor Laboratory ed.).

35 In the third approach, transcriptional activity for  
the HBMYCNG coding region can be assessed by  
hybridization assays. For example, RNA can be isolated

and analyzed by Northern blot using a probe homologous to  
5 the HBMYCNG coding sequence or particular portions  
thereof. Alternatively, total nucleic acids of the host  
cell may be extracted and assayed for hybridization to  
such probes.

In the fourth approach, the expression of the  
10 HBMYCNG protein product can be assessed immunologically,  
for example by Western blots, immunoassays such as  
radioimmuno-precipitation, enzyme-linked immunoassays and  
the like. The ultimate test of the success of the  
expression system, however, involves the detection of  
15 biologically active HBMYCNG gene product. A number of  
assays can be used to detect HBMYCNG activity including  
but not limited to binding assays and biological assays  
for HBMYCNG activity.

Once a clone that produces high levels of a  
20 biologically active HBMYCNG polypeptide is identified,  
the clone may be expanded and used to produce large  
amounts of the polypeptide which may be purified using  
techniques well known in the art, including but not  
limited to, immunoaffinity purification using antibodies,  
25 immunoprecipitation or chromatographic methods including  
high performance liquid chromatography (HPLC).

Where the HBMYCNG coding sequence is engineered to  
encode a cleavable fusion protein, purification may be  
readily accomplished using affinity purification  
30 techniques. For example, a collagenase cleavage  
recognition consensus sequence may be engineered between  
the carboxy terminus of HBMYCNG and protein A. The  
resulting fusion protein may be readily purified using an  
IgG column that binds the protein A moiety. Unfused  
35 HBMYCNG may be readily released from the column by  
treatment with collagenase. Another example would be the  
use of pGEX vectors that express foreign polypeptides as

fusion proteins with glutathione S-transferase (GST).

- 5 The fusion protein may be engineered with either thrombin or factor Xa cleavage sites between the cloned gene and the GST moiety. The fusion protein may be easily purified from cell extracts by adsorption to glutathione agarose beads followed by elution in the presence of glutathione.
- 10 In fact, any cleavage site or enzyme cleavage substrate may be engineered between the HMYCNG gene product sequence and a second peptide or protein that has a binding partner which could be used for purification, e.g., any antigen for which an immunoaffinity column can
- 15 be prepared.

In addition, HMYCNG fusion proteins may be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described by Janknecht et al. allows for the ready purification of

- 20 non-denatured fusion proteins expressed in human cell lines (Janknecht, et al., 1991, Proc. Natl. Acad. Sci. USA 88: 8972-8976). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally
- 25 fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto  $\text{Ni}^{2+}$  nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing
- 30 buffers.

In another embodiment, fusion proteins comprising at least one extracellular domain (i.e. the extracellular domains consist approximately of amino acid residues 161-173, 237-274, and 370-453) of the HMYCNG polypeptide

- 35 are expressed from a genetically-engineered gene constructed and expressed using any recombinant method described above. In one aspect of this embodiment, a

"soluble" derivative of the HMBYCNG protein is  
5 synthesized within which the six transmembrane domains  
(represented by amino acid residues 141-160, 174-192,  
217-236, 275-297, 350-369, and 454-474 of the protein  
sequence of Figure 3) are replaced with peptide sequences  
of comparable length and structure, providing a water  
10 soluble fusion protein mimic of the HMBYCNG polypeptide.

Alternatively, the HMBYCNG protein itself can be  
produced using chemical methods to synthesize the HMBYCNG  
amino acid sequence in whole or in part. For example,  
peptides can be synthesized by solid phase techniques,  
15 cleaved from the resin, and purified by preparative high  
performance liquid chromatography (see, e.g., Creighton,  
1983, Proteins Structures And Molecular Principles, W.H.  
Freeman and Co., N.Y., pp. 50-60). The composition of the  
synthetic peptides may be confirmed by amino acid  
20 analysis or sequencing (e.g., the Edman degradation  
procedure; see Creighton, 1983, Proteins, Structures and  
Molecular Principles, W.H. Freeman and Co., N.Y., pp.  
34-49).

The HMBYCNG proteins, polypeptides and peptide  
25 fragments, mutated, truncated or deleted forms of HMBYCNG  
and/or HMBYCNG fusion proteins can be prepared for  
various uses, including but not limited to, the  
generation of antibodies, as reagents in diagnostic  
assays, the identification of other cellular gene  
30 products involved in ion transport, as reagents in assays  
for screening for compounds for use in the treatment of  
ion channel disorders.

### 5.3. Antibodies to HMBYCNG Polypeptides

35 The present invention also includes antibodies  
directed to the HMBYCNG polypeptides of this invention  
and methods for the production of those antibodies,



including antibodies that specifically recognize one or  
5 more HBMYCNG epitopes or epitopes of conserved variants  
or peptide fragments of HBMYCNG, or antibodies which  
recognize the extracellular domains of the CNG  $\alpha$ -subunit  
polypeptides, or which recognize HBMYCNG epitopes within  
the water soluble fusion protein mimic of the HBMYCNG  
10 polypeptide disclosed above.

Such antibodies may include, but are not limited to,  
polyclonal antibodies, monoclonal antibodies (mAbs),  
humanized or chimeric antibodies, single chain  
antibodies, Fab fragments, F(ab')<sub>2</sub> fragments, fragments  
15 produced by a Fab expression library, anti-idiotypic  
(anti-Id) antibodies, and epitope-binding fragments of  
any of the above. Such antibodies may be used, for  
example, in the detection of a HBMYCNG protein or  
polypeptide in an biological sample and may, therefore,  
20 be utilized as part of a diagnostic or prognostic  
technique whereby patients may be tested for abnormal  
levels of HBMYCNG and/or for the presence of abnormal  
forms of the protein. Such antibodies may also be  
utilized in conjunction with, for example, compound  
25 screening protocols for the evaluation of the effect of  
test compounds on HBMYCNG levels and/or activity.  
Additionally, such antibodies can be used in conjunction  
with the gene therapy techniques described in Section  
5.4, *infra*, to, for example, evaluate normal and/or  
30 genetically-engineered HBMYCNG-expressing cells prior to  
their introduction into the patient.

An isolated polypeptide or peptide of the invention  
can be used as an immunogen to generate antibodies using  
standard techniques for polyclonal and monoclonal  
35 antibody preparation. The full-length polypeptide or a  
functional domain of the polypeptide, either native or  
denatured, can be used or, alternatively, the invention

provides antigenic polypeptides or peptides for use as  
5 immunogens. The antigenic peptide of a polypeptide of the  
invention comprises at least 8 (preferably 10, 15, 20, or  
30) amino acid residues of the amino acid sequence of SEQ  
ID NO: 2 or a variant thereof, and features an epitope of  
the polypeptide such that an antibody raised against the  
10 peptide forms a specific immune complex with the  
polypeptide, and alternatively with a native polypeptide.

Preferred epitopes encompassed by the antigenic  
peptide are regions that are located on the surface of  
the polypeptide, e.g., hydrophilic regions. In certain  
15 embodiments, the nucleic acid molecules of the invention  
are present as part of nucleic acid molecules comprising  
nucleic acid sequences that contain or encode  
heterologous (e.g., vector, expression vector, or fusion  
polypeptide) sequences. These nucleotides can then be  
20 used to express polypeptides which can be used as  
immunogens to generate an immune response, or more  
particularly, to generate polyclonal or monoclonal  
antibodies specific to the expressed polypeptide.

For the production of antibodies against HBMYCNG,  
25 various host animals may be immunized by injection with  
the protein or a portion thereof. Such host animals  
include rabbits, mice, rats, and baboons. Various  
adjuvants may be used to increase the immunological  
response, depending on the host species, including but  
30 not limited to, Freund's (complete and incomplete),  
mineral gels such as aluminum hydroxide, surface active  
substances such as lysolecithin, pluronic polyols,  
polyanions, peptides, oil emulsions, keyhole limpet  
hemocyanin, dinitrophenol, and potentially useful human  
35 adjuvants such as BCG (bacille Calmette-Guerin) and  
*Corynebacterium parvum*.

Accordingly, another aspect of the invention

5 pertains to antibodies directed against a polypeptide of the invention. The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site which specifically

10 binds an antigen, such as a polypeptide of the invention, e.g., an epitope of a polypeptide of the invention. A molecule which specifically binds to a given polypeptide of the invention is a molecule which binds the polypeptide, but does not substantially bind other

15 molecules in a sample, e.g., a biological sample, which naturally contains the polypeptide. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and F(ab')<sub>2</sub> fragments which can be generated by treating the antibody with an enzyme such as

20 pepsin. The invention provides polyclonal and monoclonal antibodies. The term "monoclonal antibody" or "monoclonal antibody composition," as used herein, refers to a population of antibody molecules that contain only one species of an antigen binding site capable of

25 immunoreacting with a particular epitope.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen, such as a HBMYCNG polypeptide, or an antigenic functional derivative thereof. For the

30 production of polyclonal antibodies, host animals such as those described above, may be immunized by injection with the HBMYCNG polypeptide supplemented with adjuvants as also described above.

Monoclonal antibodies, which are homogeneous

35 populations of antibodies to a particular antigen, may be obtained by any technique which provides for the production of antibody molecules by continuous cell lines

in culture. These include, but are not limited to, the  
 5 hybridoma technique of Kohler and Milstein (1975, Nature  
 256: 495-497; and U.S. Patent No. 4,376,110), the human  
 B-cell hybridoma technique (Kosbor et al., 1983,  
 Immunology Today 4: 72; Cole et al., 1983, Proc. Natl.  
 Acad. Sci. USA 80: 2026-2030), and the EBV-hybridoma  
 10 technique (Cole et al., 1985, Monoclonal Antibodies And  
 Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such  
 antibodies may be of any immunoglobulin class including  
 IgG, IgM, IgE, IgA, IgD and any subclass thereof. The  
 hybridomas producing the monoclonal antibodies of this  
 15 invention may be cultivated *in vitro* or *in vivo*.

In addition, techniques developed for the production  
 of chimeric antibodies (Morrison et al., 1984, Proc.  
 Natl. Acad. Sci., 81: 6851-6855; Neuberger et al., 1984,  
 Nature 312: 604-608; Takeda et al., 1985, Nature 314:  
 20 452-454) by splicing the genes from a mouse antibody  
 molecule of appropriate antigen specificity together with  
 genes from a human antibody molecule of appropriate  
 biological activity can be used. A chimeric antibody is a  
 molecule in which different portions are derived from  
 25 different animal species, such as those having a variable  
 region derived from a murine mAb and a human  
 immunoglobulin constant region (see, e.g., Cabilly et  
 al., U.S. Patent No. 4,816,567; and Boss et al., U.S.  
 Patent No. 4,816,397.)

30 The antibody titer in the immunized subject can be  
 monitored over time by standard techniques, such as with  
 an enzyme linked immunosorbent assay (ELISA) using  
 immobilized polypeptide. If desired, the antibody  
 molecules can be isolated from the mammal (e.g., from the  
 35 blood) and further purified by well-known techniques,  
 such as protein A chromatography to obtain the IgG  
 fraction. Alternatively, antibodies specific for a

polypeptide or peptide of the invention can be selected  
5 for (e.g., partially purified) or purified by, e.g.,  
affinity chromatography. For example, a recombinantly  
expressed and purified (or partially purified)  
polypeptide of the invention is produced as described  
herein, and covalently or non-covalently coupled to a  
10 solid support such as, for example, a chromatography  
column. The column can then be used to affinity purify  
antibodies specific for the polypeptides of the invention  
from a sample containing antibodies directed against a  
large number of different epitopes, thereby generating a  
15 substantially purified antibody composition, i.e., one  
that is substantially free of contaminating antibodies.  
By a substantially purified antibody composition is  
meant, in this context, that the antibody sample contains  
at most only 30% (by dry weight) of contaminating  
20 antibodies directed against epitopes other than those on  
the desired polypeptide or polypeptide of the invention,  
and preferably at most 20%, yet more preferably at most  
10%, and most preferably at most 5% (by dry weight) of  
the sample is contaminating antibodies. A purified  
25 antibody composition means that at least 99% of the  
antibodies in the composition are directed against the  
desired polypeptide or peptide of the invention.

At an appropriate time after immunization, e.g.,  
when the specific antibody titers are highest,  
30 antibody-producing cells can be obtained from the subject  
and used to prepare monoclonal antibodies by standard  
techniques, such as the hybridoma technique originally  
described by Kohler and Milstein (1975) Nature  
256:495-497, the human B cell hybridoma technique (Kozbor  
35 et al. (1983) Immunol. Today 4:72), the EBV-hybridoma  
technique (Cole et al. (1985), Monoclonal Antibodies and  
Cancer Therapy, Alan R. Liss, Inc., pp. 77-96) or trioma

techniques. The technology for producing hybridomas is  
 5 well known (see generally Current Protocols in Immunology  
 (1994) Coligan et al. (eds.) John Wiley & Sons, Inc., New  
 York, NY). Hybridoma cells producing a monoclonal  
 antibody of the invention are detected by screening the  
 hybridoma culture supernatants for antibodies that bind  
 10 the polypeptide of interest, e.g., using a standard ELISA  
 assay.

Alternative to preparing monoclonal antibody-  
 secreting hybridomas, a monoclonal antibody directed  
 against a polypeptide of the invention can be identified  
 15 and isolated by screening a recombinant combinatorial  
 immunoglobulin library (e.g., an antibody phage display  
 library) with the polypeptide of interest. Kits for  
 generating and screening phage display libraries are  
 commercially available (e.g., the Pharmacia Recombinant  
 20 Phage Antibody System, Catalog No. 27-9400-01; and the  
 Stratagene SurfZAP Phage Display Kit, Catalog No.  
 240612). Additionally, examples of methods and reagents  
 particularly amenable for use in generating and screening  
 antibody display library can be found in, for example,  
 25 U.S. Patent No. 5,223,409; PCT Publication No. WO  
 92/18619; PCT Publication No. WO 91/17271; PCT  
 Publication No. WO 92/20791; PCT Publication No. WO  
 92/15679; PCT Publication No. WO 93/01288; PCT  
 Publication No. WO 92/01047; PCT Publication No. WO  
 30 92/09690; PCT Publication No. WO 90/02809; Fuchs et al.  
 (1991) Bio/Technology 9:1370-1372; Hay et al. (1992) Hum.  
 Antibod. Hybridomas 3:81-85; Huse et al. (1989) Science  
 246:1275-1281; Griffiths et al. (1993) EMBO J.  
 12:725-734.

35 In addition, techniques have been developed for the  
 production of humanized antibodies (see, e.g., Queen,  
 U.S. Patent No. 5,585,089). Humanized antibodies are

antibody molecules from non-human species having one or  
5 more CDRs from the non-human species and a framework  
region from a human immunoglobulin molecule.

Completely human antibodies are particularly  
desirable for therapeutic treatment of human patients.  
Such antibodies can be produced, for example, using  
10 transgenic mice which are incapable of expressing  
endogenous immunoglobulin heavy and light chains genes,  
but which can express human heavy and light chain genes.  
The transgenic mice are immunized in the normal fashion  
with a selected antigen, e.g., all or a portion of a  
15 polypeptide of the invention. Monoclonal antibodies  
directed against the antigen can be obtained using  
conventional hybridoma technology. The human  
immunoglobulin transgenes harbored by the transgenic mice  
rearrange during B cell differentiation, and subsequently  
20 undergo class switching and somatic mutation. Thus, using  
such a technique, it is possible to produce  
therapeutically useful IgG, IgA and IgE antibodies. For  
an overview of this technology for producing human  
antibodies, see Lonberg and Huszar (1995, Int. Rev.  
25 Immunol. 13:65-93). For a detailed discussion of this  
technology for producing human antibodies and human  
monoclonal antibodies and protocols for producing such  
antibodies, see, e.g., U.S. Patent 5,625,126; U.S. Patent  
5,633,425; U.S. Patent 5,569,825; U.S. Patent 5,661,016;  
30 and U.S. Patent 5,545,806. In addition, companies such as  
Abgenix, Inc. (Freemont, CA), can be engaged to provide  
human antibodies directed against a selected antigen  
using technology similar to that described above.

Completely human antibodies which recognize a  
35 selected epitope can be generated using a technique  
referred to as "guided selection." In this approach a  
selected non-human monoclonal antibody, e.g., a mouse

antibody, is used to guide the selection of a completely  
5 human antibody recognizing the same epitope (Jespers et  
al. (1994) Bio/technology 12:899-903).

Alternatively, techniques described for the  
production of single chain antibodies (U.S. Patent  
4,946,778; Bird, 1988, Science 242: 423-426; Huston et  
10 al., 1988, Proc. Natl. Acad. Sci. USA 85: 5879-5883; and  
Ward et al., 1989, Nature 334: 544-546) can be used in  
the production of single chain antibodies against  
HBMYCNG. Single chain antibodies are formed by linking  
the heavy and light chain fragments of the Fv region via  
15 an amino acid bridge, resulting in a single chain  
polypeptide.

Furthermore, antibody fragments which recognize  
specific epitopes of HBMYCNG may be produced by  
techniques well known in the art. For example, such  
20 fragments include but are not limited to,  $F(ab')_2$   
fragments which can be produced by pepsin digestion of  
the antibody molecule and Fab fragments which can be  
generated by reducing the disulfide bridges of the  $F(ab')_2$   
fragments. Alternatively, Fab expression libraries may be  
25 constructed (Huse et al., 1989, Science 246: 1275-1281)  
to allow rapid and easy identification of monoclonal Fab  
fragments with the desired specificity.

An antibody directed against a polypeptide of the  
invention (e.g., monoclonal antibody) can be used to  
30 isolate the polypeptide by standard techniques, such as  
affinity chromatography or immunoprecipitation. Moreover,  
such an antibody can be used to detect the polypeptide  
(e.g., in a cellular lysate or cell supernatant) in order  
to evaluate the abundance and pattern of expression of  
35 the polypeptide. The antibodies can also be used  
diagnostically to monitor polypeptide levels in tissue as  
part of a clinical testing procedure, e.g., to, for



- example, determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials.
- 10 Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase,  $\beta$ -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials
- 15 include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin,
- 20 and aequorin, and examples of suitable radioactive material include  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{35}\text{S}$ ,  $^{99}\text{Tc}$  or  $^3\text{H}$ .

- In addition, the HBMYCNG gene sequences and gene products, including polypeptides, peptides, fusion polypeptides or peptides, and antibodies directed against
- 25 said gene products and peptides, have applications for purposes independent of the role of the gene products. For example, HBMYCNG gene products, including polypeptides or peptides, as well as specific antibodies thereto, can be used for construction of fusion
- 30 polypeptides to facilitate recovery, detection, or localization of another polypeptide of interest. In addition, HBMYCNG genes and gene products can be used for genetic mapping. Finally, HBMYCNG nucleic acids and gene products have generic uses, such as supplemental sources
- 35 of nucleic acids, polypeptides and amino acids for food additives or cosmetic products.

Further, an antibody of the invention (or fragment thereof) may be conjugated to a therapeutic moiety such as a cytotoxin, a therapeutic agent or a radioactive metal ion. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine and vinblastine).

In addition, polypeptides, agonists or antagonists which bind a polypeptide of the invention can also be conjugated to the foregoing, thereby targeting a toxin to cells expressing HGPRBM1.

The conjugates of the invention can be used for modifying a given biological response, the drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a polypeptide or peptide possessing a desired biological activity. Such polypeptides may include, for example, a

- toxin such as abrin, ricin A, pseudomonas exotoxin, or
- 5 diphtheria toxin; a polypeptide such as tumor necrosis factor,  $\gamma$ -interferon,  $\alpha$ -interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator, a thrombotic agent or an anti-angiogenic agent, e.g., angiostatin or endostatin; or, biological
- 10 response modifiers such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-4 ("IL-4"), interleukin-6 ("IL-6"), interleukin-7 ("IL-7"), granulocyte macrophage colony stimulating factor ("GM-CSF"), granulocyte colony
- 15 stimulating factor ("G-CSF"), interleukin-10 ("IL-10"), interleukin-12 ("IL-12"), interleukin-17 ("IL-15"), interleukin-17 ("IL-17"), interferon- $\gamma$  ("IFN- $\gamma$ "), interferon- $\alpha$  ("IFN- $\alpha$ "), or other immune factors or growth factors.
- 20 Techniques for conjugating such therapeutic moiety to antibodies are well known, see, e.g., Arnon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in Monoclonal Antibodies And Cancer Therapy, Reisfeld et al. (eds.), pp. 243-56 (Alan R.
- 25 Liss, Inc. 1985); Hellstrom et al., "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd Ed.), Robinson et al. (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in Monoclonal Antibodies:
- 30 Biological And Clinical Applications, Pinchera et al. (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in Monoclonal Antibodies For Cancer Detection And Therapy, Baldwin et al. (eds.), pp.
- 35 303-16 (Academic Press 1985), and Thorpe et al., "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", Immunol. Rev., 62:119-58 (1982).

An antibody with or without a therapeutic moiety  
5 conjugated to it can be used as a therapeutic that is  
administered alone or in combination with  
chemotherapeutic agents.

Alternatively, an antibody of the invention can be  
conjugated to a second antibody to form an "antibody  
10 heteroconjugate" as described by Segal in U.S. Patent No.  
4,676,980 or alternatively, the antibodies can be  
conjugated to form an "antibody heteropolymer" as  
described in Taylor et al., in U.S. Patent Nos. 5,470,570  
and 5,487,890.

15 An antibody with or without a therapeutic moiety  
conjugated to it can be used as a therapeutic that is  
administered alone or in combination with cytotoxic  
factor(s) and/or cytokine(s).

In yet a further aspect, the invention provides  
20 substantially purified antibodies or fragments thereof,  
including human or non-human antibodies or fragments  
thereof, which antibodies or fragments specifically bind  
to a polypeptide of the invention comprising an amino  
acid sequence of SEQ ID NO: 2 or a variant thereof. In  
25 various embodiments, the substantially purified  
antibodies of the invention, or fragments thereof, can be  
human, non-human, chimeric and/or humanized antibodies.

In another aspect, the invention provides human or  
non-human antibodies or fragments thereof, which  
30 antibodies or fragments specifically bind to a  
polypeptide comprising an amino acid sequence of SEQ ID  
NO: 2 or a variant thereof. Such non-human antibodies can  
be goat, mouse, sheep, horse, chicken, rabbit, or rat  
antibodies. Alternatively, the non-human antibodies of  
35 the invention can be chimeric and/or humanized  
antibodies. In addition, the non-human antibodies of the

invention can be polyclonal antibodies or monoclonal  
5 antibodies.

In still a further aspect, the invention provides  
monoclonal antibodies or fragments thereof, which  
antibodies or fragments specifically bind to a  
polypeptide of the invention comprising an amino acid  
10 sequence of SEQ ID NO: 2 or a variant thereof. The  
monoclonal antibodies can be human, humanized, chimeric  
and/or non-human antibodies.

The substantially purified antibodies or fragments  
thereof specifically bind to a signal peptide, a secreted  
15 sequence, an extracellular domain, a transmembrane or a  
cytoplasmic domain of a polypeptide of the invention. In  
a particularly preferred embodiment, the substantially  
purified antibodies or fragments thereof, the non-human  
antibodies or fragments thereof, and/or the monoclonal  
20 antibodies or fragments thereof, of the invention  
specifically bind to a secreted sequence, or  
alternatively, to an extracellular domain of the amino  
acid sequence of the invention.

Any of the antibodies of the invention can be  
25 conjugated to a therapeutic moiety or to a detectable  
substance. Non-limiting examples of detectable substances  
that can be conjugated to the antibodies of the invention  
are an enzyme, a prosthetic group, a fluorescent  
material, a luminescent material, a bioluminescent  
30 material, and a radioactive material.

The invention also provides a kit containing an  
antibody of the invention conjugated to a detectable  
substance, and instructions for use. Still another aspect  
of the invention is a pharmaceutical composition  
35 comprising an antibody of the invention and a  
pharmaceutically acceptable carrier. In preferred  
embodiments, the pharmaceutical composition contains an

antibody of the invention, a therapeutic moiety, and a  
 5 pharmaceutically acceptable carrier.

Still another aspect of the invention is a method of  
 making an antibody that specifically recognizes HBMYCNG,  
 the method comprising immunizing a mammal with a  
 polypeptide. After immunization, a sample is collected  
 10 from the mammal that contains an antibody that  
 specifically recognizes the immunogen. Preferably, the  
 polypeptide is recombinantly produced using a non-human  
 host cell. Optionally, the antibodies can be further  
 purified from the sample using techniques well known to  
 15 those of skill in the art. The method can further  
 comprise producing a monoclonal antibody-producing cell  
 from the cells of the mammal. Optionally, antibodies are  
 collected from the antibody-producing cell.

#### 20 5.4. Uses of the HBMYCNG Nucleic Acid Molecules, Gene Products, and Antibodies

As discussed *supra*, the HBMYCNG gene of this  
 invention encodes a protein involved in the formation or  
 function of ion channels, more particularly, cation  
 25 channels. Given the importance of cations such as  
 calcium, sodium or potassium in many cellular processes,  
 the HBMYCNG nucleic acid molecules and polypeptides of  
 this invention are useful for the diagnosis and treatment  
 of a variety of human disease conditions which involve  
 30 ion, more particularly, cation, channel dysfunction.

For example, calcium plays a role in the release of  
 neurotransmitters, hormones and other circulating  
 factors, the expression of numerous regulatory genes as  
 well as the cellular process of apoptosis or cell death.  
 35 Potassium provides for neuroprotection and also affects  
 insulin secretion. Sodium is involved in the regulation  
 of normal neuronal action potential generation and

propagation. Sodium channel blockers such as lidocaine  
5 are important analgesics. Therefore, cation channel  
dysfunction may play a role in many human diseases and  
disorders such as CNS disorders, e.g., stroke, anxiety,  
and depression, Alzheimer's disease, or Parkinson's  
disease, and other diseases such as cardiac disorders,  
10 e.g., arrhythmia, diabetes, chronic pain, hypercalcemia,  
hypercalciuria, or ion channel dysfunction that is  
associated with immunological disorders,  
gastro-intestinal (GI) tract disorders, or renal or liver  
disease. Moreover, modulation of calcium transport may  
15 play a role in the proper functioning of the serotonin  
nervous system which also participates in the control of  
anxiety, fear, depression, sleep and pain. Accordingly,  
cation channel dysfunction may further play a role in  
anxiety and fear disorders, bipolar and major depression,  
20 panic disorder, headaches, migraine, disorders of  
circadian rhythmicity, stress, various sexual  
dysfunctions including but not limited to erectile  
dysfunction, neuroleptic-induced catalepsy, Rett syndrome  
and aggressive behaviors. As such, proteins that are  
25 involved in either the formation or function of these ion  
channels (and the nucleic acids that encode those  
proteins) are useful for the diagnosis and treatment of  
many human diseases.

Among the uses for the nucleic acid molecules and  
30 polypeptides of the invention are the prognostic and  
diagnostic evaluation of human disorders involving  
ion/cation channel dysfunction, and the identification of  
subjects with a predisposition to such disorders, as  
described below. Other uses include methods for the  
35 treatment of such ion/cation channel dysfunction  
disorders, for the modulation of HBMYCNG gene-mediated

activity, and for the modulation of HBMYCNG-mediated  
 5 effector functions.

In addition, the nucleic acid molecules and  
 polypeptides of the invention can be used in assays for  
 the identification of compounds which modulate the  
 expression of the HBMYCNG genes of the invention and/or  
 10 the activity of the HBMYCNG gene products. Such compounds  
 can include, for example, other cellular products or  
 small molecule compounds that are involved in cation  
 homeostasis or activity.

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5 5.4.1. Diagnosis and Prognosis of Ion-Related Disorders

Methods of the invention for the diagnosis and prognosis of human diseases involving ion, e.g., cation, dysfunction may utilize reagents such as the HBMYCNG nucleic acid molecules and sequences described in Sections 5.1, *supra*, or antibodies directed against HBMYCNG polypeptides, including peptide fragments thereof, as described in Section 5.3., *supra*. Specifically, such reagents may be used, for example, for: (1) the detection of the presence of HBMYCNG gene mutations, or the detection of either over- or under-expression of HBMYCNG gene mRNA relative to the non-cation dysfunctional state or the qualitative or quantitative detection of alternatively spliced forms of HBMYCNG transcripts which may correlate with certain ion homeostasis disorders or susceptibility toward such disorders; and (2) the detection of either an over- or an under-abundance of HBMYCNG gene product relative to the non- cation dysfunctional state or the presence of a modified (e.g., less than full length) HBMYCNG gene product which correlates with a cation dysfunctional state or a progression toward such a state.

The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic test kits comprising at least one specific HBMYCNG gene nucleic acid or anti-HBMYCNG gene antibody reagent described herein, which may be conveniently used, e.g., in clinical settings, to screen and diagnose patients exhibiting ion/cation channel/homeostasis abnormalities and to screen and identify those individuals exhibiting a predisposition to such abnormalities.

For the detection of HBMYCNG mutations, any  
 5 nucleated cell can be used as a starting source for  
 genomic nucleic acid. For the detection of HBMYCNG  
 transcripts or HBMYCNG gene products, any cell type or  
 tissue in which the HBMYCNG gene is expressed may be  
 utilized.

10 Nucleic acid-based detection techniques are  
 described in Section 5.4.1.1., *infra*, whereas  
 peptide-based detection techniques are described in  
 Section 5.4.1.2., *infra*.

#### 15 5.4.1.1. Detection of Hbmycng Gene Nucleic Acid Molecules

Mutations or polymorphisms within the HBMYCNG gene  
 can be detected by utilizing a number of techniques.  
 Nucleic acid from any nucleated cell can be used as the  
 20 starting point for such assay techniques, and may be  
 isolated according to standard nucleic acid preparation  
 procedures which are well known to those of skill in the  
 art.

Genomic DNA may be used in hybridization or  
 25 amplification assays of biological samples to detect  
 abnormalities involving HBMYCNG gene structure, including  
 point mutations, insertions, deletions and chromosomal  
 rearrangements. Such assays may include, but are not  
 limited to, direct sequencing (Wong, C. et al., 1987,  
 30 Nature 330:384-386), single stranded conformational  
 polymorphism analyses (SSCP; Orita, M. et al., 1989,  
 Proc. Natl. Acad. Sci. USA 86:2766-2770), heteroduplex  
 analysis (Keen, T.J. et al., 1991, Genomics 11:199-205;  
 Perry, D.J. & Carrell, R.W., 1992), denaturing gradient  
 35 gel electrophoresis (DGGE; Myers, R.M. et al., 1985,  
 Nucl. Acids Res. 13:3131-3145), chemical mismatch  
 cleavage (Cotton, R.G. et al., 1988, Proc. Natl. Acad.

- Sci. USA 85:4397-4401) and oligonucleotide hybridization  
5 (Wallace, R.B. et al., 1981, Nucl. Acids Res. 9:879-894;  
Lipshutz, R.J. et al., 1995, Biotechniques 19:442-447).

Diagnostic methods for the detection of HBMYCNG gene  
specific nucleic acid molecules, in patient samples or  
other appropriate cell sources, may involve the  
10 amplification of specific gene sequences, e.g., by PCR,  
followed by the analysis of the amplified molecules using  
techniques well known to those of skill in the art, such  
as, for example, those listed above. Utilizing analysis  
techniques such as these, the amplified sequences can be  
15 compared to those which would be expected if the nucleic  
acid being amplified contained only normal copies of the  
HBMYCNG gene in order to determine whether a HBMYCNG gene  
mutation exists.

Further, well-known genotyping techniques can be  
20 performed to type polymorphisms that are in close  
proximity to mutations in the HBMYCNG gene itself. These  
polymorphisms can be used to identify individuals in  
families likely to carry mutations. If a polymorphism  
exhibits linkage disequilibrium with mutations in the  
25 HBMYCNG gene, it can also be used to identify individuals  
in the general population likely to carry mutations.  
Polymorphisms that can be used in this way include  
restriction fragment length polymorphisms (RFLPs), which  
involve sequence variations in restriction enzyme target  
30 sequences, single-base polymorphisms and simple sequence  
repeat polymorphisms (SSLPs).

For example, Weber (U.S. Pat. No. 5,075,217)  
describes a DNA marker based on length polymorphisms in  
blocks of (dC-dA)<sub>n</sub>-(dG-dT)<sub>n</sub> short tandem repeats. The  
35 average separation of (dC-dA)<sub>n</sub>-(dG-dT)<sub>n</sub> blocks is  
estimated to be 30,000-60,000 bp. Markers which are so  
closely spaced exhibit a high frequency co-inheritance,

and are extremely useful in the identification of genetic mutations, such as, for example, mutations within the HBMYCNG gene, and the diagnosis of diseases and disorders related to HBMYCNG mutations.

Also, Caskey et al. (U.S. Pat.No. 5,364,759) describe a DNA profiling assay for detecting short tri- and tetra- nucleotide repeat sequences. The process includes extracting the DNA of interest, such as the HBMYCNG gene, amplifying the extracted DNA, and labelling the repeat sequences to form a genotypic map of the individual's DNA.

A HBMYCNG probe could additionally be used to directly identify RFLPs. Additionally, a HBMYCNG probe or primers derived from the HBMYCNG sequences of the invention could be used to isolate genomic clones such as YACs, BACs, PACs, cosmids, phage or plasmids. The DNA contained in these clones can be screened for single-base polymorphisms or simple sequence length polymorphisms (SSLPs) using standard hybridization or sequencing procedures.

Alternative diagnostic methods for the detection of HBMYCNG gene-specific mutations or polymorphisms can include hybridization techniques which involve for example, contacting and incubating nucleic acids including recombinant DNA molecules, cloned genes or degenerate variants thereof, obtained from a sample, e.g., derived from a patient sample or other appropriate cellular source, with one or more labeled nucleic acid reagents including the HBMYCNG nucleic acid molecules of the invention including recombinant DNA molecules, cloned genes or degenerate variants thereof, as described in Section 5.1 *supra*, under conditions favorable for the specific annealing of these reagents to their complementary sequences within the HBMYCNG gene.

Preferably, the lengths of these nucleic acid reagents  
5 are at least 15 to 30 nucleotides. After incubation, all  
non-annealed nucleic acids are removed from the nucleic  
acid:HBMYCNG molecule hybrid. The presence of nucleic  
acids which have hybridized, if any such molecules exist,  
is then detected. Using such a detection scheme, the  
10 nucleic acid from the cell type or tissue of interest can  
be immobilized, for example, to a solid support such as a  
membrane, or a plastic surface such as that on a  
microtiter plate or polystyrene beads. In this case,  
after incubation, non-annealed, labeled nucleic acid  
15 molecules of the invention as described in Section 5.1  
are easily removed. Detection of the remaining, annealed,  
labeled HBMYCNG nucleic acid reagents is accomplished  
using standard techniques well-known to those in the art.  
The HBMYCNG gene sequences to which the nucleic acid  
20 molecules of the invention have annealed can be compared  
to the annealing pattern expected from a normal HBMYCNG  
gene sequence in order to determine whether a HBMYCNG  
gene mutation is present.

Quantitative and qualitative aspects of HBMYCNG gene  
25 expression can also be assayed. For example, RNA from a  
cell type or tissue known, or suspected, to express the  
HBMYCNG gene may be isolated and tested utilizing  
hybridization or PCR techniques as described *supra*. The  
isolated cells can be derived from cell culture or from a  
30 patient. The analysis of cells taken from culture may be  
a necessary step in the assessment of cells to be used as  
part of a cell-based gene therapy technique or,  
alternatively, to test the effect of compounds on the  
expression of the HBMYCNG gene. Such analyses may reveal  
35 both quantitative and qualitative aspects of the  
expression pattern of the HBMYCNG gene, including

activation or inactivation of HBMYCNG gene expression and  
5 presence of alternatively spliced HBMYCNG transcripts.

In one embodiment of such a detection scheme, a cDNA  
molecule is synthesized from an RNA molecule of interest  
(e.g., by reverse transcription of the RNA molecule into  
cDNA). All or part of the resulting cDNA is then used as  
10 the template for a nucleic acid amplification reaction,  
such as a PCR amplification reaction, or the like. The  
nucleic acid reagents used as synthesis initiation  
reagents (e.g., primers) in the reverse transcription and  
nucleic acid amplification steps of this method are  
15 chosen from among the HBMYCNG nucleic acid molecules of  
the invention as described in Section 5.1, *supra*. The  
preferred lengths of such nucleic acid reagents are at  
least 9-30 nucleotides.

For detection of the amplified product, the nucleic  
20 acid amplification may be performed using radioactively  
or non-radioactively labeled nucleotides. Alternatively,  
enough amplified product may be made such that the  
product may be visualized by standard ethidium bromide  
staining or by utilizing any other suitable nucleic acid  
25 staining method.

Such RT-PCR techniques can be utilized to detect  
differences in HBMYCNG transcript size which may be due  
to normal or abnormal alternative splicing. Additionally,  
such techniques can be utilized to detect quantitative  
30 differences between levels of full length and/or  
alternatively spliced HBMYCNG transcripts detected in  
normal individuals relative to those individuals  
exhibiting ion dysfunction disorders or exhibiting a  
predisposition to toward such disorders.

35 In the case where detection of specific  
alternatively spliced species is desired, appropriate  
primers and/or hybridization probes can be used, such

that, in the absence of such sequence, no amplification  
 5 would occur. Alternatively, primer pairs may be chosen  
 utilizing the sequences depicted in FIG. 1, 3 or 5 to  
 choose primers which will yield fragments of differing  
 size depending on whether a particular exon is present or  
 absent from the HBMYCNG transcript being utilized.

10 As an alternative to amplification techniques,  
 standard Northern analyses can be performed if a  
 sufficient quantity of the appropriate cells can be  
 obtained. Utilizing such techniques, quantitative as well  
 as size-related differences between HBMYCNG transcripts  
 15 can also be detected.

Additionally, it is possible to perform HBMYCNG gene  
 expression assays *in situ*, i.e., directly upon tissue  
 sections (fixed and/or frozen) of patient tissue obtained  
 from biopsies or resections, such that no nucleic acid  
 20 purification is necessary. The nucleic acid molecules of  
 the invention as described in Section 5.1 may be used as  
 probes and/or primers for such *in situ* procedures (see,  
 for example, Nuovo, G.J., 1992, "PCR In Situ  
 Hybridization: Protocols And Applications", Raven Press,  
 25 NY).

#### 5.4.1.2. Detection of HBMYCNG Gene Products

Antibodies directed against wild type or mutant  
 HBMYCNG gene products or conserved variants or peptide  
 30 fragments or extracellular domain thereof as described  
*supra* may also be used for the diagnosis and prognosis of  
 ion or cation-related disorders. Such diagnostic methods  
 may be used to detect abnormalities in the level of  
 HBMYCNG gene expression or abnormalities in the structure  
 35 and/or temporal, tissue, cellular, or subcellular  
 location of HBMYCNG gene products. Antibodies, or  
 fragments of antibodies, may be used to screen

- potentially therapeutic compounds *in vitro* to determine
- 5 their effects on HBMYCNG gene expression and HBMYCNG peptide production. The compounds which have beneficial effects on ion and cation-related disorders can be identified and a therapeutically effective dose determined.
- 10 *In vitro* immunoassays may be used, for example, to assess the efficacy of cell-based gene therapy for ion or cation-related disorders. For example, antibodies directed against HBMYCNG peptides may be used *in vitro* to determine the level of HBMYCNG gene expression achieved
- 15 in cells genetically engineered to produce HBMYCNG peptides. Such analysis will allow for a determination of the number of transformed cells necessary to achieve therapeutic efficacy *in vivo*, as well as optimization of the gene replacement protocol.
- 20 The tissue or cell type to be analyzed will generally include those which are known, or suspected, to express the HBMYCNG gene. The protein isolation methods employed may, for example, be such as those described in Harlow, E. and Lane, D., 1988, "Antibodies: A Laboratory
- 25 Manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. The isolated cells can be derived from cell culture or from a patient. The analysis of cells taken from culture may be a necessary step in the assessment of cells to be used as part of a cell-based
- 30 gene therapy technique or, alternatively, to test the effect of compounds on the expression of the HBMYCNG gene.

Preferred diagnostic methods for the detection of HBMYCNG gene products or conserved variants or peptide

35 fragments thereof, may involve, for example, immunoassays wherein the HBMYCNG gene products or conserved variants, including gene products which are the result of



alternatively spliced transcripts, or peptide fragments  
5 are detected by their interaction with an anti-HBMYCNG  
gene product-specific antibody.

For example, antibodies, or fragments of antibodies,  
such as those described in Section 5.3 *supra*, may be used  
to quantitatively or qualitatively detect the presence of  
10 HBMYCNG gene products or conserved variants or peptide  
fragments thereof. The antibodies (or fragments thereof)  
may, additionally, be employed histologically, as in  
immunofluorescence or immunoelectron microscopy, for *in situ*  
detection of HBMYCNG gene products or conserved  
15 variants or peptide fragments thereof. *In situ* detection  
may be accomplished by removing a histological specimen  
from a patient, and applying thereto a labeled HBMYCNG  
antibody of the present invention. The antibody (or  
fragment) is preferably applied by overlaying the labeled  
20 antibody (or fragment) onto a biological sample. Through  
the use of such a procedure, it is possible to determine  
not only the presence of the HBMYCNG gene product, or  
conserved variants or peptide fragments, but also its  
distribution in the examined tissue. Using the present  
25 invention, those of ordinary skill will readily perceive  
that any of a wide variety of histological methods (such  
as staining procedures) can be modified in order to  
achieve such *in situ* detection.

Immunoassays for HBMYCNG gene products or conserved  
30 variants or peptide fragments thereof will typically  
comprise incubating a sample, such as a biological fluid,  
a tissue extract, freshly harvested cells, or lysates of  
cells which have been incubated in cell culture, in the  
presence of a detectably labeled antibody capable of  
35 identifying HBMYCNG gene products or conserved variants  
or peptide fragments thereof, and detecting the bound

antibody by any of a number of techniques well-known in  
5 the art.

The biological sample may be brought in contact with  
and immobilized onto a solid phase support or carrier  
such as nitrocellulose, or other solid support which is  
capable of immobilizing cells, cell particles or soluble  
10 proteins. The support may then be washed with suitable  
buffers followed by treatment with the detectably labeled  
HBMYCNG gene specific antibody. The solid phase support  
may then be washed with the buffer a second time to  
remove unbound antibody. The amount of bound label on  
15 solid support may then be detected by conventional means.

By "solid phase support or carrier" is intended any  
support capable of binding an antigen or an antibody.  
Well-known supports or carriers include glass,  
polystyrene, polypropylene, polyethylene, dextran, nylon,  
20 amylases, natural and modified celluloses,  
polyacrylamides, gabbros, and magnetite. The nature of  
the carrier can be either soluble to some extent or  
insoluble. The support material may have virtually any  
possible structural configuration so long as the coupled  
25 molecule is capable of binding to an antigen or antibody.  
Thus, the support configuration may be spherical, as in a  
bead, or cylindrical, as in the inside surface of a test  
tube, or the external surface of a rod. Alternatively,  
the surface may be flat such as a sheet, test strip, etc.  
30 Preferred supports include polystyrene beads. Those  
skilled in the art will know many other suitable carriers  
for binding antibody or antigen, or will be able to  
ascertain the same by use of routine experimentation.

The binding activity of a given lot of anti-HBMYCNG  
35 gene product antibody may be determined according to well  
known methods. Those skilled in the art will be able to

determine operative and optimal assay conditions for each  
 5 determination by employing routine experimentation.

One of the ways in which the HBMYCNG gene  
 peptide-specific antibody can be detectably labeled is by  
 linking the antibody to an enzyme in an enzyme  
 immunoassay (EIA) (Voller, A., "The Enzyme Linked  
 10 Immunosorbent Assay (ELISA)", 1978, Diagnostic Horizons  
 2:1-7, Microbiological Associates Quarterly Publication,  
 Walkersville, MD); Voller, A. et al., 1978, J. Clin.  
 Pathol. 31:507-520; Butler, J.E., 1981, Meth. Enzymol.  
 73:482-523; Maggio, E. (ed.), 1980, Enzyme Immunoassay,  
 15 CRC Press, Boca Raton, FL; Ishikawa, E. et al., (eds.),  
 1981, Enzyme Immunoassay, Kigaku Shoin, Tokyo). The enzyme  
 which is bound to the antibody will react with an  
 appropriate substrate, preferably a chromogenic  
 substrate, in such a manner as to produce a chemical  
 20 moiety which can be detected, for example, by  
 spectrophotometric, fluorimetric or by visual means.  
 Enzymes which can be used to detectably label the  
 antibody include, but are not limited to, malate  
 dehydrogenase, staphylococcal nuclease, delta-5-steroid  
 25 isomerase, yeast alcohol dehydrogenase,  
 alpha-glycerophosphate, dehydrogenase, triose phosphate  
 isomerase, horseradish peroxidase, alkaline phosphatase,  
 asparaginase, glucose oxidase, beta-galactosidase,  
 ribonuclease, urease, catalase, glucose-6-phosphate  
 30 dehydrogenase, glucoamylase and acetylcholinesterase. The  
 detection can be accomplished by colorimetric methods  
 which employ a chromogenic substrate for the enzyme.  
 Detection may also be accomplished by visual comparison  
 of the extent of enzymatic reaction of a substrate in  
 35 comparison with similarly prepared standards.

Detection may also be accomplished using any of a  
 variety of other immunoassays. For example, by

radioactively labeling the antibodies or antibody  
 5 fragments, it is possible to detect HBMYCNG gene peptides  
 through the use of a radioimmunoassay (RIA) (see, for  
 example, Weintraub, B., Principles of Radioimmunoassays,  
 Seventh Training Course on Radioligand Assay Techniques,  
 The Endocrine Society, March, 1986. The radioactive  
 10 isotope can be detected by such means as the use of a  
 gamma counter or a scintillation counter or by  
 autoradiography.

It is also possible to label the antibody with a  
 fluorescent compound. When the fluorescently labeled  
 15 antibody is exposed to light of the proper wave length,  
 its presence can then be detected due to fluorescence.  
 Among the most commonly used fluorescent labeling  
 compounds are fluorescein isothiocyanate, rhodamine,  
 phycoerythrin, phycocyanin, allophycocyanin,  
 20 o-phthalaldehyde and fluorescamine.

The antibody can also be detectably labeled using  
 fluorescence emitting metals such as  $^{152}\text{Eu}$ , or others of  
 the lanthanide series. These metals can be attached to  
 the antibody using such metal chelating groups as  
 25 diethylenetriaminepentacetic acid (DTPA) or  
 ethylenediaminetetraacetic acid (EDTA).

The antibody also can be detectably labeled by  
 coupling it to a chemiluminescent compound. The presence  
 of the chemiluminescent-tagged antibody is then  
 30 determined by detecting the presence of luminescence that  
 arises during the course of a chemical reaction. Examples  
 of particularly useful chemiluminescent labeling  
 compounds are luminol, isoluminol, theromatic acridinium  
 ester, imidazole, acridinium salt and oxalate ester.

35 Likewise, a bioluminescent compound may be used to  
 label the antibody of the present invention.  
 Bioluminescence is a type of chemiluminescence found in

biological systems in which a catalytic protein increases  
 5 the efficiency of the chemiluminescent reaction. The  
 presence of a bioluminescent protein is determined by  
 detecting the presence of luminescence. Important  
 bioluminescent compounds for purposes of labeling are  
 luciferin, luciferase and aequorin.

10

#### 5.4.2. Screening Assays for Compounds That Modulate HBMYCNG Activity

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Screening assays can be used to identify compounds  
 that modulate HBMYCNG activity. These compounds can  
 15 include, but are not limited to, peptides, small organic  
 or inorganic molecules or macromolecules such as nucleic  
 acid molecules or proteins, and may be utilized, e.g., in  
 the control of ion and cation-related disorders, in the  
 modulation of cellular processes such as the release of  
 20 neurotransmitters or other cellular regulatory factors,  
 cell activation or regulation, cell death and changes in  
 cell membrane properties. These compounds may also be  
 useful, e.g., in elaborating the biological functions of  
 HBMYCNG gene products, modulating those biological  
 25 functions and for ameliorating symptoms of ion or  
 cation-related disorders.

The compositions of the invention include  
 pharmaceutical compositions comprising one or more of  
 these compounds. Such pharmaceutical compositions can be  
 30 formulated as discussed in Section 5.5, *infra*.

More specifically, these compounds can include  
 compounds that bind to HBMYCNG gene products, compounds  
 that bind to other proteins that interact with a HBMYCNG  
 gene product and/or interfere with the interaction of the  
 35 HBMYCNG gene product with other proteins, and compounds  
 that modulate the activity of the HBMYCNG gene, i.e.,

- modulate the level of HBMYCNG gene expression and/or
- 5 modulate the level of HBMYCNG gene product activity.

For example, assays may be utilized that identify compounds that bind to HBMYCNG gene regulatory sequences, e.g., promoter sequences (see e.g., Platt, K.A., 1994, J. Biol. Chem. 269:28558-28562), which compounds may

- 10 modulate the level of HBMYCNG gene expression. In addition, functional assays can be used to screen for compounds that modulate HBMYCNG gene product activity. In such assays, compounds are screened for agonistic or antagonistic activity with respect to a biological
- 15 activity or function of the HBMYCNG gene product, such as changes in the intracellular levels of an ion or cation, changes in regulatory factor release, or other activities or functions of the HBMYCNG polypeptides of the invention.

- 20 According to a preferred embodiment, a  $\text{Ca}^{2+}$  flux assay can be utilized to monitor calcium uptake in HBMYCNG-expressing host cells. The host cells are pre-loaded with a  $\text{Ca}^{2+}$ -sensitive fluorescently-labeled dye (e.g., Fluo-4, Fluo-3, Indo-1 or Fura-2), i.e., the
- 25 intracellular calcium is fluorescently labelled with the dye, and the effect of the compound, e.g., on the intracellular levels of the labeled-calcium determined and compared to the intracellular levels of control cells, e.g., lacking exposure to the compound of
- 30 interest. Compounds that have an agonistic, i.e., stimulatory, modulatory effect on HBMYCNG activity are those that, when contacted with the HBMYCNG-expressing cells, produce an increase in intracellular calcium relative to the control cells, whereas those compounds
- 35 having an antagonistic modulatory effect on HBMYCNG activity will be those that block the effects of agonists or cyclic nucleotides that increase channel activity. A

Ca<sup>2+</sup> flux assay is exemplified in Example Section 6.1,  
5 *infra*.

Functional assays for monitoring the effects of compounds on the levels or flux of other ions can be similarly performed; for example, the levels of potassium can be monitored using rubidium influx.

10 Screening assays may also be designed to identify compounds capable of binding to the HBMYCNG gene products of the invention. Such compounds may be useful, e.g., in modulating the activity of wild type and/or mutant HBMYCNG gene products, in elaborating the biological  
15 function of the HBMYCNG gene product, and in screens for identifying compounds that disrupt normal HBMYCNG gene product interactions, or may in themselves disrupt such interactions.

The principle of such screening assays to identify  
20 compounds that bind to the HBMYCNG gene product involves preparing a reaction mixture of the HBMYCNG gene product and the test compound under conditions and for a time sufficient to allow the two components to interact with, i.e., bind to, and thus form a complex, which can  
25 represent a transient complex, which can be removed and/or detected in the reaction mixture. For example, one assay involves anchoring a HBMYCNG gene product or the test substance onto a solid phase and detecting HBMYCNG gene product/test compound complexes anchored on the  
30 solid phase at the end of the reaction. In one embodiment of such a method, the HBMYCNG gene product may be anchored onto a solid surface, and the test compound, which is not anchored, may be labeled, either directly or indirectly.

35 The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the previously non-immobilized component is pre-labeled,

the detection of label immobilized on the surface  
 5 indicates that complexes were formed. Where the  
 previously non-immobilized component is not pre-labeled,  
 an indirect label can be used to detect complexes  
 anchored on the surface; e.g., using a labeled antibody  
 specific for the previously non-immobilized component  
 10 (the antibody, in turn, may be directly labeled or  
 indirectly labeled with a labeled anti-Ig antibody).

Alternatively, a reaction can be conducted in a  
 liquid phase, the reaction products separated from  
 unreacted components, and complexes detected; e.g., using  
 15 an immobilized antibody specific for HBMYCNG gene product  
 or the test compound to anchor any complexes formed in  
 solution, and a labeled antibody specific for the other  
 component of the possible complex to detect anchored  
 complexes.

20 Compounds that modulate HBMYCNG gene product  
 activity can also include compounds that bind to proteins  
 that interact with the HBMYCNG gene product. These  
 modulatory compounds can be identified by first  
 identifying those proteins that interact with the HBMYCNG  
 25 gene product, e.g., by standard techniques known in the  
 art for detecting protein-protein interactions, such as  
 co-immunoprecipitation, crosslinking and co-purification  
 through gradients or chromatographic columns. Utilizing  
 procedures such as these allows for the isolation of  
 30 proteins that interact with HBMYCNG gene products or  
 polypeptides of the invention as described *supra*.

Once isolated, such a protein can be identified and  
 can, in turn, be used, in conjunction with standard  
 techniques, to identify additional proteins with which it  
 35 interacts. For example, at least a portion of the amino  
 acid sequence of the protein that interacts with the  
 HBMYCNG gene product can be ascertained using techniques



well known to those of skill in the art, such as via the  
 5 Edman degradation technique (see, e.g., Creighton, 1983,  
 "Proteins: Structures and Molecular Principles", W.H.  
 Freeman & Co., N.Y., pp.34-49). The amino acid sequence  
 thus obtained may be used as a guide for the generation  
 of oligonucleotide mixtures that can be used to screen  
 10 for gene sequences encoding such proteins. Screening may  
 be accomplished, for example, by standard hybridization  
 or PCR techniques. Techniques for the generation of  
 oligonucleotide mixtures and screening are well-known  
 (see, e.g., Ausubel, *supra.*, and PCR Protocols: A Guide  
 15 to Methods and Applications, 1990, Innis, M. et al., eds.  
 Academic Press, Inc., New York).

Additionally, methods may be employed that result in  
 the simultaneous identification of genes which encode  
 proteins interacting with HBMYCNG gene products or  
 20 polypeptides. These methods include, for example, probing  
 expression libraries with labeled HBMYCNG protein, using  
 HBMYCNG protein in a manner similar to the well known  
 technique of antibody probing of  $\lambda$ gt11 libraries. One  
 method that detects protein interactions *in vivo* is the  
 25 two-hybrid system. A version of this system is described  
 by Chien et al., 1991, Proc. Natl. Acad. Sci. USA,  
 88:9578-9582 and is commercially available from Clontech  
 (Palo Alto, CA).

In addition, compounds that disrupt HBMYCNG  
 30 interactions with its interacting or binding partners, as  
 determined immediately above, may be useful in regulating  
 the activity of the HBMYCNG gene product, including  
 mutant HBMYCNG gene products. Such compounds may include,  
 but are not limited to molecules such as peptides, and  
 35 the like, which may bind to the HBMYCNG gene product as  
 described above.

The basic principle of the assay systems used to  
5 identify compounds that interfere with the interaction  
between the HBMYCNG gene product and its interacting  
partner or partners involves preparing a reaction mixture  
containing the HBMYCNG gene product, and the interacting  
partner under conditions and for a time sufficient to  
10 allow the two to interact and bind, thus forming a  
complex. In order to test a compound for inhibitory  
activity, the reaction mixture is prepared in the  
presence and absence of the test compound. The test  
compound may be initially included in the reaction  
15 mixture, or may be added at a time subsequent to the  
addition of HBMYCNG gene product and its interacting  
partner. Control reaction mixtures are incubated without  
the test compound or with a placebo. The formation of any  
complexes between the HBMYCNG gene product and the  
20 interacting partner is then detected. The formation of a  
complex in the control reaction, but not in the reaction  
mixture containing the test compound, indicates that the  
compound interferes with the interaction of the HBMYCNG  
gene product and the interacting partner. Additionally,  
25 complex formation within reaction mixtures containing the  
test compound and a normal HBMYCNG gene product may also  
be compared to complex formation within reaction mixtures  
containing the test compound and a mutant HBMYCNG gene  
product. This comparison may be important in those cases  
30 wherein it is desirable to identify compounds that  
disrupt interactions of mutant but not normal HBMYCNG  
proteins.

The assay for compounds that interfere with the  
interaction of HBMYCNG gene products and interacting  
35 partners can be conducted in a heterogeneous or  
homogeneous format. Heterogeneous assays involve  
anchoring either the HBMYCNG gene product or the binding

partner onto a solid phase and detecting complexes  
5 anchored on the solid phase at the end of the reaction.  
In homogeneous assays, the entire reaction is carried out  
in a liquid phase. In either approach, the order of  
addition of reactants can be varied to obtain different  
information about the compounds being tested. For  
10 example, test compounds that interfere with the  
interaction between the HBMYCNG gene products and the  
interacting partners, e.g., by competition, can be  
identified by conducting the reaction in the presence of  
the test substance; i.e., by adding the test substance to  
15 the reaction mixture prior to or simultaneously with the  
HBMYCNG gene product and interacting partner.

Alternatively, test compounds that disrupt preformed  
complexes, e.g., compounds with higher binding constants  
that displace one of the components from the complex, can  
20 be tested by adding the test compound to the reaction  
mixture after complexes have been formed. The various  
formats are described briefly below.

In a heterogeneous assay system, either the HBMYCNG  
gene product or the interacting partner, is anchored onto  
25 a solid surface, while the non-anchored species is  
labeled, either directly or indirectly. In practice,  
microtiter plates are conveniently utilized. The anchored  
species may be immobilized by non-covalent or covalent  
attachments. Non-covalent attachment may be accomplished  
30 simply by coating the solid surface with a solution of  
the HBMYCNG gene product or interacting partner and  
drying. Alternatively, an immobilized antibody specific  
for the species to be anchored may be used to anchor the  
species to the solid surface. The surfaces may be  
35 prepared in advance and stored.

In order to conduct the assay, the partner of the  
immobilized species is exposed to the coated surface with

- or without the test compound. After the reaction is
- 5 complete, unreacted components are removed (e.g., by washing) and any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the non-immobilized species is pre-labeled,
- 10 the detection of label immobilized on the surface indicates that complexes were formed. Where the non-immobilized species is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; e.g., using a labeled antibody specific for the
- 15 initially non-immobilized species (the antibody, in turn, may be directly labeled or indirectly labeled with a labeled anti-Ig antibody). Depending upon the order of addition of reaction components, test compounds which inhibit complex formation or which disrupt preformed
- 20 complexes can be detected.

Alternatively, the reaction can be conducted in a liquid phase in the presence or absence of the test compound, the reaction products separated from unreacted components, and complexes detected; e.g., using an

25 immobilized antibody specific for one of the interacting components to anchor any complexes formed in solution, and a labeled antibody specific for the other partner to detect anchored complexes. Again, depending upon the order of addition of reactants to the liquid phase, test

30 compounds that inhibit complex formation or that disrupt preformed complexes can be identified.

In an alternate embodiment, a preformed complex of the HBMYCNG gene protein and the interacting partner is prepared in which either the HBMYCNG gene product or its

35 interacting partners is labeled, but the signal generated by the label is quenched due to complex formation (see, e.g., U.S. Patent No. 4,109,496 by Rubenstein which

utilizes this approach for immunoassays). The addition of  
5 a test substance that competes with and displaces one of  
the species from the preformed complex will result in the  
generation of a signal above background. In this way,  
test substances that disrupt HBMYCNG gene  
protein/interacting partner interaction can be  
10 identified.

In another embodiment of the invention, these same  
techniques can be employed using peptide fragments that  
correspond to the binding domains of the HBMYCNG protein  
and/or the interacting partner, in place of one or both  
15 of the full length proteins. Any number of methods  
routinely practiced in the art can be used to identify  
and isolate the binding sites. These methods include, but  
are not limited to, mutagenesis of the gene encoding one  
of the proteins and screening for disruption of binding  
20 in a co-immunoprecipitation assay. Compensating mutations  
in the gene encoding the second species in the complex  
can then be selected. Sequence analysis of the genes  
encoding the respective proteins will reveal the  
mutations that correspond to the region of the protein  
25 involved in interacting, e.g., binding. Alternatively,  
one protein can be anchored to a solid surface using  
methods described in this Section above, and allowed to  
interact with, e.g., bind, to its labeled interacting  
partner, which has been treated with a proteolytic  
30 enzyme, such as trypsin. After washing, a short, labeled  
peptide comprising the interacting, e.g., binding, domain  
may remain associated with the solid material, which can  
be isolated and identified by amino acid sequencing.  
Also, once the gene coding for the intracellular binding  
35 partner is obtained, short gene segments can be  
engineered to express peptide fragments of the protein,

which can then be tested for binding activity and  
5 purified or synthesized.

The human HBMYCNG polypeptides and/or peptides of  
the present invention, or immunogenic fragments or  
oligopeptides thereof, can be used for screening  
therapeutic drugs or compounds in a variety of drug  
10 screening techniques. The fragment employed in such a  
screening assay may be free in solution, affixed to a  
solid support, borne on a cell surface, or located  
intracellularly. The reduction or abolition of activity  
of the formation of binding complexes between the ion  
15 channel protein and the agent being tested can be  
measured. Thus, the present invention provides a method  
for screening or assessing a plurality of compounds for  
their specific binding affinity with a HBMYCNG  
polypeptide, or a bindable peptide fragment, of this  
20 invention, comprising providing a plurality of compounds,  
combining the HBMYCNG polypeptide, or a bindable peptide  
fragment, with each of a plurality of compounds for a  
time sufficient to allow binding under suitable  
conditions and detecting binding of the HBMYCNG  
25 polypeptide or peptide to each of the plurality of test  
compounds, thereby identifying the compounds that  
specifically bind to the HBMYCNG polypeptide or peptide.

Methods of identifying compounds that modulate the  
activity of the novel human HBMYCNG polypeptides and/or  
30 peptides are provided by the present invention and  
comprise combining a potential or candidate compound or  
drug modulator of ion channel biological activity with an  
HBMYCNG polypeptide or peptide, for example, the HBMYCNG  
amino acid sequence as set forth in SEQ ID NOS:2, and  
35 measuring an effect of the candidate compound or drug  
modulator on the biological activity of the HBMYCNG  
polypeptide or peptide. Such measurable effects include,  
for example, physical binding interaction; the ability to

cleave a suitable ion channel substrate; effects on  
5 native and cloned HBMYCNG-expressing cell line; and  
effects of modulators or other ion channel-mediated  
physiological measures.

Another method of identifying compounds that  
modulate the biological activity of the novel HBMYCNG  
10 polypeptides of the present invention comprises combining  
a potential or candidate compound or drug modulator of a  
ion channel biological activity with a host cell that  
expresses the HBMYCNG polypeptide and measuring an effect  
of the candidate compound or drug modulator on the  
15 biological activity of the HBMYCNG polypeptide. The host  
cell can also be capable of being induced to express the  
HBMYCNG polypeptide, e.g., via inducible expression.  
Physiological effects of a given modulator candidate on  
the HBMYCNG polypeptide can also be measured. Thus,  
20 cellular assays for particular ion channel modulators may  
be either direct measurement or quantification of the  
physical biological activity of the HBMYCNG polypeptide,  
or they may be measurement or quantification of a  
physiological effect. Such methods preferably employ a  
25 HBMYCNG polypeptide as described herein, or an  
overexpressed recombinant HBMYCNG polypeptide in suitable  
host cells containing an expression vector as described  
herein, wherein the HBMYCNG polypeptide is expressed,  
overexpressed, or undergoes upregulated expression.

Another aspect of the present invention embraces a  
30 method of screening for a compound that is capable of  
modulating the biological activity of a HBMYCNG  
polypeptide, comprising providing a host cell containing  
an expression vector harboring a nucleic acid sequence  
35 encoding a HBMYCNG polypeptide, or a functional peptide  
or portion thereof (e.g., SEQ ID NOS:2); determining the  
biological activity of the expressed HBMYCNG polypeptide  
in the absence of a modulator compound; contacting the

- cell with the modulator compound and determining the
- 5 biological activity of the expressed HBMYCNG polypeptide in the presence of the modulator compound. In such a method, a difference between the activity of the HBMYCNG polypeptide in the presence of the modulator compound and in the absence of the modulator compound indicates a
- 10 modulating effect of the compound.

- Essentially any chemical compound can be employed as a potential modulator or ligand in the assays according to the present invention. Compounds tested as ion channel modulators can be any small chemical compound, or
- 15 biological entity (e.g., protein, sugar, nucleic acid, lipid). Test compounds will typically be small chemical molecules and peptides. Generally, the compounds used as potential modulators can be dissolved in aqueous or organic (e.g., DMSO-based) solutions. The assays are
- 20 designed to screen large chemical libraries by automating the assay steps and providing compounds from any convenient source. Assays are typically run in parallel, for example, in microtiter formats on microtiter plates in robotic assays. There are many suppliers of chemical
- 25 compounds, including Sigma (St. Louis, MO), Aldrich (St. Louis, MO), Sigma-Aldrich (St. Louis, MO), Fluka Chemika-Biochemica Analytika (Buchs, Switzerland), for example. Also, compounds may be synthesized by methods known in the art.

- 30 High throughput screening methodologies are particularly envisioned for the detection of modulators of the novel HBMYCNG polynucleotides and polypeptides described herein. Such high throughput screening methods typically involve providing a combinatorial chemical or
- 35 peptide library containing a large number of potential therapeutic compounds (e.g., ligand or modulator compounds). Such combinatorial chemical libraries or ligand libraries are then screened in one or more assays



to identify those library members (e.g., particular  
5 chemical species or subclasses) that display a desired  
characteristic activity. The compounds so identified can  
serve as conventional lead compounds, or can themselves  
be used as potential or actual therapeutics.

A combinatorial chemical library is a collection of  
10 diverse chemical compounds generated either by chemical  
synthesis or biological synthesis, by combining a number  
of chemical building blocks (i.e., reagents such as amino  
acids). As an example, a linear combinatorial library,  
e.g., a polypeptide or peptide library, is formed by  
15 combining a set of chemical building blocks in every  
possible way for a given compound length (i.e., the  
number of amino acids in a polypeptide or peptide  
compound). Millions of chemical compounds can be  
synthesized through such combinatorial mixing of chemical  
20 building blocks.

The preparation and screening of combinatorial  
chemical libraries is well known to those having skill in  
the pertinent art. Combinatorial libraries include,  
without limitation, peptide libraries (e.g. U.S. Patent  
25 No. 5,010,175; Furka, 1991, *Int. J. Pept. Prot. Res.*,  
37:487-493; and Houghton et al., 1991, *Nature*, 354:84-  
88). Other chemistries for generating chemical diversity  
libraries can also be used. Nonlimiting examples of  
chemical diversity library chemistries include, peptoids  
30 (PCT Publication No. WO 91/019735), encoded peptides (PCT  
Publication No. WO 93/20242), random bio-oligomers (PCT  
Publication No. WO 92/00091), benzodiazepines (U.S.  
Patent No. 5,288,514), diversomers such as hydantoins,  
benzodiazepines and dipeptides (Hobbs et al., 1993, *Proc.*  
35 *Natl. Acad. Sci. USA*, 90:6909-6913), vinylogous  
polypeptides (Hagihara et al., 1992, *J. Amer. Chem. Soc.*,  
114:6568), nonpeptidal peptidomimetics with glucose  
scaffolding (Hirschmann et al., 1992, *J. Amer. Chem.*

- Soc., 114:9217-9218), analogous organic synthesis of
- 5 small compound libraries (Chen et al., 1994, *J. Amer. Chem. Soc.*, 116:2661), oligocarbamates (Cho et al., 1993, *Science*, 261:1303), and/or peptidyl phosphonates (Campbell et al., 1994, *J. Org. Chem.*, 59:658), nucleic acid libraries (see Ausubel, Berger and Sambrook, all
- 10 supra), peptide nucleic acid libraries (U.S. Patent No. 5,539,083), antibody libraries (e.g., Vaughn et al., 1996, *Nature Biotechnology*, 14(3):309-314) and PCT/US96/10287), carbohydrate libraries (e.g., Liang et al., 1996, *Science*, 274-1520-1522) and U.S. Patent No.
- 15 5,593,853), small organic molecule libraries (e.g., benzodiazepines, Baum C&EN, Jan. 18, 1993, page 33; and U.S. Patent No. 5,288,514; isoprenoids, U.S. Patent No. 5,569,588; thiazolidinones and metathiazanones, U.S. Patent No. 5,549,974; pyrrolidines, U.S. Patent Nos.
- 20 5,525,735 and 5,519,134; morpholino compounds, U.S. Patent No. 5,506,337; and the like).

- Devices for the preparation of combinatorial libraries are commercially available (e.g., 357 MPS, 390 MPS, Advanced Chem Tech, Louisville KY; Symphony, Rainin,
- 25 Woburn, MA; 433A Applied Biosystems, Foster City, CA; 9050 Plus, Millipore, Bedford, MA). In addition, a large number of combinatorial libraries are commercially available (e.g., ComGenex, Princeton, NJ; Asinex, Moscow, Russia; Tripos, Inc., St. Louis, MO; ChemStar, Ltd.,
- 30 Moscow, Russia; 3D Pharmaceuticals, Exton, PA; Martek Biosciences, Columbia, MD, and the like).

- In one embodiment, the invention provides solid phase based *in vitro* assays in a high throughput format, where the cell or tissue expressing an ion channel is
- 35 attached to a solid phase substrate. In such high throughput assays, it is possible to screen up to several thousand different modulators or ligands in a single day. In particular, each well of a microtiter plate can be

used to perform a separate assay against a selected  
5 potential modulator, or, if concentration or incubation  
time effects are to be observed, every 5-10 wells can  
test a single modulator. Thus, a single standard  
microtiter plate can assay about 96 modulators. If 1536  
10 well plates are used, then a single plate can easily  
assay from about 100 to about 1500 different compounds.  
It is possible to assay several different plates per day;  
thus, for example, assay screens for up to about 6,000-  
20,000 different compounds are possible using the  
described integrated systems.

15 In another of its aspects, the present invention  
encompasses screening and small molecule (e.g., drug)  
detection assays which involve the detection or  
identification of small molecules that can bind to a  
given protein, i.e., a HBMYCNG polypeptide or peptide.  
20 Particularly preferred are assays suitable for high  
throughput screening methodologies.

In such binding-based detection, identification, or  
screening assays, a functional assay is not typically  
required. All that is needed is a target protein,  
25 preferably substantially purified, and a library or panel  
of compounds (e.g., ligands, drugs, small molecules) or  
biological entities to be screened or assayed for binding  
to the protein target. Preferably, most small molecules  
that bind to the target protein will modulate activity in  
30 some manner, due to preferential, higher affinity binding  
to functional areas or sites on the protein.

An example of such an assay is the fluorescence  
based thermal shift assay (3-Dimensional Pharmaceuticals,  
Inc., 3DP, Exton, PA) as described in U.S. Patent Nos.  
6,020,141 and 6,036,920 to Pantoliano et al.; see also,  
35 J. Zimmerman, 2000, *Gen. Eng. News*, 20(8)). The assay  
allows the detection of small molecules (e.g., drugs,  
ligands) that bind to expressed, and preferably purified,

ion channel polypeptide based on affinity of binding  
5 determinations by analyzing thermal unfolding curves of  
protein-drug or ligand complexes. The drugs or binding  
molecules determined by this technique can be further  
assayed, if desired, by methods, such as those described  
herein, to determine if the molecules affect or modulate  
10 function or activity of the target protein.

To purify a HBMYCNG polypeptide or peptide to  
measure a biological binding or ligand binding activity,  
the source may be a whole cell lysate that can be  
prepared by successive freeze-thaw cycles (e.g., one to  
15 three) in the presence of standard protease inhibitors.  
The HBMYCNG polypeptide may be partially or completely  
purified by standard protein purification methods, e.g.,  
affinity chromatography using specific antibody described  
*infra*, or by ligands specific for an epitope tag  
20 engineered into the recombinant HBMYCNG polypeptide  
molecule, also as described herein. Binding activity can  
then be measured as described.

Compounds which are identified according to the  
methods provided herein, and which modulate or regulate  
25 the biological activity or physiology of the HBMYCNG  
polypeptides according to the present invention are a  
preferred embodiment of this invention. It is  
contemplated that such modulatory compounds may be  
employed in treatment and therapeutic methods for  
30 treating a condition that is mediated by the novel  
HBMYCNG polypeptides by administering to an individual in  
need of such treatment a therapeutically effective amount  
of the compound identified by the methods described  
herein.

In addition, the present invention provides methods  
35 for treating an individual in need of such treatment for  
a disease, disorder, or condition that is mediated by the  
HBMYCNG polypeptides of the invention, comprising

- administering to the individual a therapeutically  
 5 effective amount of the HBMYCNG-modulating compound  
 identified by a method provided herein.

#### 5.4.3. Methods and Compositions for the Treatment of Ion Channel-Related Disorders

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- 10 The present invention also relates to methods and  
 compositions for the treatment or modulation of any  
 disorder or cellular process that is mediated or  
 regulated by HBMYCNG gene product expression or function,  
 e.g., HBMYCNG-mediated cell activation, signal  
 15 transduction, cellular regulatory factor release, etc.  
 Further, HBMYCNG effector functions can be modulated via  
 such methods and compositions.

- The methods of the invention include methods that  
 modulate HBMYCNG gene and gene product activity. In  
 20 certain instances, the treatment will require an  
 increase, upregulation or activation of HBMYCNG activity,  
 while in other instances, the treatment will require a  
 decrease, downregulation or suppression of HBMYCNG  
 activity. "Increase" and "decrease" refer to the  
 25 differential level of HBMYCNG activity relative to  
 HBMYCNG activity in the cell type of interest in the  
 absence of modulatory treatment. Methods for the decrease  
 of HBMYCNG activity are discussed in Section 5.4.3.1,  
*infra*. Methods for the increase of HBMYCNG activity are  
 30 discussed in Section 5.4.3.2, *infra*. Methods which can  
 either increase or decrease HBMYCNG activity depending on  
 the particular manner in which the method is practiced  
 are discussed in Section 5.4.3.3, *infra*.

##### 35 5.4.3.1. Methods for Decreasing HBMYCNG Activity

Successful treatment of ion channel/ionic  
 homeostasis disorders, e.g., CNS disorders, cardiac

disorders or hypercalcemia, can be brought about by  
5 methods which serve to decrease HBMYCNG activity.  
Activity can be decreased by, e.g., directly decreasing  
HBMYCNG gene product activity and/or by decreasing the  
level of HBMYCNG gene expression.

For example, compounds such as those identified  
10 through assays described in Section 5.4.2., *supra*, that  
decrease HBMYCNG gene product activity can be used in  
accordance with the invention to ameliorate symptoms  
associated with ion channel/ionic homeostasis disorders.  
As discussed *supra*, such molecules can include, but are  
15 not limited to peptides, including soluble peptides, and  
small organic or inorganic molecules, and can be referred  
to as HBMYCNG antagonists. Techniques for the  
determination of effective doses and administration of  
such compounds are described in Section 5.5., *infra*.

20 In addition, antisense and ribozyme molecules that  
inhibit HBMYCNG gene expression can also be used to  
reduce the level of HBMYCNG gene expression, thus  
effectively reducing the level of HBMYCNG gene product  
present, thereby decreasing the level of HBMYCNG  
25 activity. Still further, triple helix molecules can be  
utilized in reducing the level of HBMYCNG gene  
expression. Such molecules can be designed to reduce or  
inhibit either wild type, or if appropriate, mutant  
target gene activity. Techniques for the production and  
30 use of such molecules are well known to those of skill in  
the art.

Antisense approaches involve the design of  
oligonucleotides (either DNA or RNA) that are  
complementary to HBMYCNG gene mRNA. The antisense  
35 oligonucleotides will bind to the complementary HBMYCNG  
gene mRNA transcripts and prevent translation. Absolute  
complementarity, although preferred, is not required. A

sequence "complementary" to a portion of an RNA, as  
5 referred to herein, means a sequence having sufficient  
complementarity to be able to hybridize with the RNA,  
forming a stable duplex; in the case of double-stranded  
antisense nucleic acids, a single strand of the duplex  
DNA may thus be tested, or triplex formation may be  
10 assayed. The ability to hybridize will depend on both the  
degree of complementarity and the length of the antisense  
nucleic acid. Generally, the longer the hybridizing  
nucleic acid, the more base mismatches with an RNA it may  
contain and still form a stable duplex (or triplex, as  
15 the case may be). One skilled in the art can ascertain a  
tolerable degree of mismatch by use of standard  
procedures to determine the melting point of the  
hybridized complex.

Oligonucleotides that are complementary to the 5'  
20 end of the message, e.g., the 5' untranslated sequence up  
to and including the AUG initiation codon, should work  
most efficiently at inhibiting translation. However,  
sequences complementary to the 3' untranslated sequences  
of mRNAs have recently been shown to be effective at  
25 inhibiting translation of mRNAs as well. See generally,  
Wagner, R., 1994, Nature 372:333-335. Thus,  
oligonucleotides complementary to either the 5'- or 3'-  
non-translated, non-coding regions of the HBMYCNG gene,  
as depicted in FIG. 1 could be used in an antisense  
30 approach to inhibit translation of endogenous HBMYCNG  
gene mRNA.

Oligonucleotides complementary to the 5'  
untranslated region of the mRNA should include the  
complement of the AUG start codon. Antisense  
35 oligonucleotides complementary to mRNA coding regions are  
less efficient inhibitors of translation but could be  
used in accordance with the invention. Whether designed

to hybridize to the 5'-, 3'- or coding region of target  
 5 or pathway gene mRNA, antisense nucleic acids should be  
 at least six nucleotides in length, and are preferably  
 oligonucleotides ranging from 6 to about 50 nucleotides  
 in length. In specific aspects, the oligonucleotide is at  
 least 10 nucleotides, at least 17 nucleotides, at least  
 10 25 nucleotides or at least 50 nucleotides.

Regardless of the choice of target sequence, it is  
 preferred that *in vitro* studies are first performed to  
 quantitate the ability of the antisense oligonucleotide  
 to inhibit gene expression. It is preferred that these  
 15 studies utilize controls that distinguish between  
 antisense gene inhibition and non-specific biological  
 effects of oligonucleotides. It is also preferred that  
 these studies compare levels of the target RNA or protein  
 with that of an internal control RNA or protein.  
 20 Additionally, results obtained using the antisense  
 oligonucleotide are preferably compared with those  
 obtained using a control oligonucleotide. It is preferred  
 that the control oligonucleotide is of approximately the  
 same length as the antisense oligonucleotide and that the  
 25 nucleotide sequence of the control oligonucleotide  
 differs from the antisense sequence no more than is  
 necessary to prevent specific hybridization to the target  
 sequence.

The oligonucleotides can be DNA or RNA or chimeric  
 30 mixtures or derivatives or modified versions thereof,  
 single-stranded or double-stranded. The oligonucleotide  
 can be modified at the base moiety, sugar moiety, or  
 phosphate backbone, for example, to improve stability of  
 the molecule, hybridization, etc.

35 The oligonucleotide may also include other appended  
 groups such as peptides (e.g., for targeting host cell  
 receptors *in vivo*), or agents facilitating transport



across the cell membrane (see, e.g., Letsinger et al.,  
5 1989, Proc. Natl. Acad. Sci. U.S.A. 86:6553-6556;  
Lemaitre et al., 1987, Proc. Natl. Acad. Sci. 84:648-652;  
PCT Application No.  
WO 88/09810) or the blood-brain barrier (see, e.g., PCT  
Application No. WO 89/10134), or hybridization-triggered  
10 cleavage agents (see, e.g., Krol et al., 1988,  
BioTechniques 6:958-976) or intercalating agents (see,  
e.g., Zon, 1988, Pharm. Res. 5:539-549). For example, the  
oligonucleotide may be conjugated to another molecule,  
e.g., a peptide, hybridization triggered cross-linking  
15 agent, transport agent, hybridization-triggered cleavage  
agent, etc.

Oligonucleotides of the invention may be synthesized  
by standard methods known in the art, e.g., by use of an  
automated DNA synthesizer (such as are commercially  
20 available from Biosearch, Applied Biosystems, etc.). As  
examples, phosphorothioate oligonucleotides may be  
synthesized by the method of Stein et al. (1988, Nucl.  
Acids Res. 16:3209) and methylphosphonate  
oligonucleotides can be prepared by use of controlled  
25 pore glass polymer supports (Sarin et al., 1988, Proc.  
Natl. Acad. Sci. U.S.A. 85:7448-7451), etc.

The antisense molecules should be delivered to cells  
which express the HBMYCNG gene *in vivo*. A number of  
methods have been developed for delivering antisense DNA  
30 or RNA to cells; e.g., antisense molecules can be  
injected directly into the tissue site or modified  
antisense molecules designed to target the desired cells  
(e.g., antisense linked to peptides or antibodies that  
specifically bind receptors or antigens expressed on the  
35 target cell surface) can be administered systemically.

However, it is often difficult to achieve  
intracellular concentrations of the antisense sufficient

to suppress translation of endogenous mRNAs. Thus, a  
 5 preferred approach utilizes a recombinant DNA construct  
 in which the antisense oligonucleotide is placed under  
 the control of a strong pol III or pol II promoter. The  
 use of such a construct to transfect target cells in the  
 patient will result in the transcription of sufficient  
 10 amounts of single stranded RNAs that will form  
 complementary base pairs with the endogenous HBMYCNG gene  
 transcripts and thereby prevent translation of the  
 HBMYCNG gene mRNA. For example, a vector can be  
 introduced *in vivo* such that it is taken up by a cell and  
 15 directs the transcription of an antisense RNA.

Ribozymes are enzymatic RNA molecules capable of  
 catalyzing the specific cleavage of RNA (For a review,  
 see, e.g., Rossi, J., 1994, Current Biology 4:469-471).  
 The mechanism of ribozyme action involves  
 20 sequence-specific hybridization of the ribozyme molecule  
 to complementary target RNA, followed by a  
 endonucleolytic cleavage. The composition of ribozyme  
 molecules must include one or more sequences  
 complementary to the target gene mRNA, and must include  
 25 the well known catalytic sequence responsible for mRNA  
 cleavage. For this sequence, see United States Patent No.  
 5,093,246, which is incorporated by reference herein in  
 its entirety. As such, within the scope of the invention  
 are engineered hammerhead motif ribozyme molecules that  
 30 specifically and efficiently catalyze endonucleolytic  
 cleavage of RNA sequences encoding target gene proteins.

Ribozyme molecules designed to catalytically cleave  
 HBMYCNG gene mRNA transcripts can also be used to prevent  
 translation of HBMYCNG gene mRNA and expression of target  
 35 or pathway genes. (See, e.g., PCT Application No. WO  
 90/11364; Sarver et al., 1990, Science 247:1222-1225).

The ribozymes of the present invention also include  
5 RNA endoribonucleases (hereinafter referred to as  
"Cech-type ribozymes") such as the one which occurs  
naturally in *Tetrahymena Thermophila* (known as the IVS,  
or L-19 IVS RNA) and which has been extensively described  
by Thomas Cech and collaborators (Zaug, et al., 1984,  
10 Science, 224:574-578; Zaug and Cech, 1986, Science,  
231:470-475; Zaug, et al., 1986, Nature, 324:429-433; PCT  
Patent Application No. WO 88/04300; Been and Cech, 1986,  
Cell, 47:207-216). The Cech-type ribozymes have an eight  
base pair active site which hybridizes to a target RNA  
15 sequence, after which cleavage of the target RNA takes  
place. The invention encompasses those Cech-type  
ribozymes which target eight base-pair active site  
sequences that are present in an HBMYCNG gene.

As in the antisense approach, the ribozymes can be  
20 composed of modified oligonucleotides (e.g. for improved  
stability, targeting, etc.) and should be delivered to  
cells which express the HBMYCNG gene *in vivo*. A preferred  
method of delivery involves using a DNA construct  
"encoding" the ribozyme under the control of a strong  
25 constitutive pol III or pol II promoter, so that  
transfected cells will produce sufficient quantities of  
the ribozyme to destroy endogenous HBMYCNG gene messages  
and inhibit translation. Because ribozymes, unlike  
antisense molecules, are catalytic, a lower intracellular  
30 concentration is required for efficiency.

Endogenous HBMYCNG gene expression can also be  
reduced by inactivating or "knocking out" the target  
and/or pathway gene or its promoter using targeted  
homologous recombination (see, e.g., Smithies et al.,  
35 1985, Nature 317:230-234; Thomas & Capecchi, 1987, Cell  
51:503-512; Thompson et al., 1989 Cell 5:313-321). For  
example, a mutant, non-functional HBMYCNG gene (or a

- completely unrelated DNA sequence) flanked by DNA
- 5 homologous to the endogenous HBMYCNG gene (either the coding regions or regulatory regions of the HBMYCNG gene) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express the HBMYCNG gene *in vivo*. Insertion of the DNA
  - 10 construct, via targeted homologous recombination, results in inactivation of the HBMYCNG gene. Such techniques can also be utilized to generate ion/cation disorder animal models. It should be noted that this approach can be adapted for use in humans provided the recombinant DNA
  - 15 constructs are directly administered or targeted to the required site *in vivo* using appropriate viral vectors, e.g., herpes virus vectors.

- Alternatively, endogenous HBMYCNG gene expression can be reduced by targeting deoxyribonucleotide sequences
- 20 complementary to the regulatory region of the HBMYCNG gene (i.e., the HBMYCNG gene promoter and/or enhancers) to form triple helical structures that prevent transcription of the HBMYCNG gene in target cells in the body (see generally, Helene, C., 1991, *Anticancer Drug*
  - 25 *Des.* 6(6):569-84; Helene, C., et al., 1992, *Ann. N.Y. Acad. Sci.* 660:27-36; and Maher, L.J., 1992, *Bioassays* 14(12):807-15).

- Nucleic acid molecules to be used in triple helix formation for the inhibition of transcription should be
- 30 single stranded and composed of deoxynucleotides. The base composition of these oligonucleotides should be designed to promote triple helix formation via Hoogsteen base pairing rules, which generally require sizeable stretches of either purines or pyrimidines to be present
  - 35 on one strand of the duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC+ triplets across the three associated strands of the

resulting triple helix. The pyrimidine-rich molecules  
5 provide base complementarity to a purine-rich region of a  
single strand of the duplex in a parallel orientation to  
that strand. In addition, nucleic acid molecules may be  
chosen that are purine-rich, for example, containing a  
stretch of G residues. These molecules will form a triple  
10 helix with a DNA duplex that is rich in GC pairs, in  
which the majority of the purine residues are located on  
a single strand of the targeted duplex, resulting in GGC  
triplets across the three strands of the triplex.

Alternatively, the potential sequences that can be  
15 targeted for triple helix formation may be increased by  
creating a "switchback" nucleic acid molecule. Switchback  
molecules are synthesized in an alternating 5'-3', 3'-5'  
manner, such that they base pair with first one strand of  
a duplex and then the other, eliminating the necessity  
20 for a sizeable stretch of either purines or pyrimidines  
to be present on one strand of the duplex.

In instances wherein the antisense, ribozyme, and/or  
triple helix molecules described herein are utilized to  
inhibit mutant HBMYCNG gene expression, it is possible  
25 that the technique may so efficiently reduce or inhibit  
the transcription (triple helix) and/or translation  
(antisense, ribozyme) of mRNA produced by normal target  
gene alleles that the concentration of normal target gene  
product present may be lower than is necessary for a  
30 normal phenotype. In such cases, to ensure that  
substantially normal levels of HBMYCNG gene activity are  
maintained, nucleic acid molecules that encode and  
express HBMYCNG gene polypeptides exhibiting normal  
target gene activity can be introduced into cells via  
35 gene therapy methods that do not contain sequences  
susceptible to whatever antisense, ribozyme, or triple  
helix treatments are being utilized. In instances where

the target gene encodes an extracellular protein, it can  
 5 be preferable to coadminister normal target gene protein  
 in order to maintain the requisite level of target gene  
 activity.

Antisense RNA and DNA, ribozyme, and triple helix  
 molecules of the invention can be prepared by any method  
 10 known in the art, e.g., methods for chemically  
 synthesizing oligodeoxyribonucleotides and  
 oligoribonucleotides well known in the art such as solid  
 phase phosphoramidite chemical synthesis. Alternatively,  
 RNA molecules can be generated by *in vitro* and *in vivo*  
 15 transcription of DNA sequences encoding the antisense RNA  
 molecule. Such DNA sequences can be incorporated into a  
 wide variety of vectors which incorporate suitable RNA  
 polymerase promoters such as the T7 or SP6 polymerase  
 promoters. Alternatively, antisense cDNA constructs that  
 20 synthesize antisense RNA constitutively or inducibly,  
 depending on the promoter used, can be introduced stably  
 into cell lines.

In addition, well-known modifications to DNA  
 molecules can be introduced into the HBMYCNG nucleic acid  
 25 molecules of the invention as a means of increasing  
 intracellular stability and half-life. Possible  
 modifications include, but are not limited to, the  
 addition of flanking sequences of ribo- or deoxy-  
 nucleotides to the 5' and/or 3' ends of the molecule or  
 30 the use of phosphorothioate or 2' O-methyl rather than  
 phosphodiesterase linkages within the  
 oligodeoxyribonucleotide backbone.

#### 5.4.3.2. Methods for Increasing HBMYCNG Activity

35 Successful treatment of ion/cation disorders can  
 also be brought about by techniques which serve to  
 increase the level of HBMYCNG activity. Activity can be

increased by, for example, directly increasing HBMYCNG  
 5 gene product activity and/or by increasing the level of  
 HBMYCNG gene expression.

For example, compounds such as those identified  
 through the assays described in Section 5.4.2., *supra*,  
 that increase HBMYCNG activity can be used to treat  
 10 ion/cation-related disorders. Such molecules can include,  
 but are not limited to peptides, including soluble  
 peptides, and small organic or inorganic molecules, and  
 can be referred to as HBMYCNG agonists.

For example, a compound can, at a level sufficient  
 15 to treat ion/cation-related disorders and symptoms, be  
 administered to a patient exhibiting such symptoms. One  
 of skill in the art will readily know how to determine  
 the concentration of effective, non-toxic doses of the  
 compound, utilizing techniques such as those described  
 20 *infra*.

Alternatively, in instances wherein the compound to  
 be administered is a peptide compound, DNA sequences  
 encoding the peptide compound can be directly  
 administered to a patient exhibiting an  
 25 ion/cation-related disorder or symptoms, at a  
 concentration sufficient to produce a level of peptide  
 compound sufficient to ameliorate the symptoms of the  
 disorder. Any of the techniques discussed *infra*, which  
 achieve intracellular administration of compounds, such  
 30 as, for example, liposome administration, can be utilized  
 for the administration of such DNA molecules. In the case  
 of peptide compounds which act extracellularly, the DNA  
 molecules encoding such peptides can be taken up and  
 expressed by any cell type, so long as a sufficient  
 35 circulating concentration of peptide results for the  
 elicitation of a reduction in the ion/cation disorder  
 symptoms.

In cases where the ion/cation disorder can be localized to a particular portion or region of the body, the DNA molecules encoding such modulatory peptides may be administered as part of a delivery complex. Such a delivery complex can comprise an appropriate nucleic acid molecule and a targeting means. Such targeting means can comprise, for example, sterols lipids, viruses or target cell specific binding agents. Viral vectors can include, but are not limited to adenovirus, adeno-associated virus, and retrovirus vectors, in addition to other particles that introduce DNA into cells, such as liposomes.

Further, in instances wherein the ion/cation-related disorder involves an aberrant HBMYCNG gene, patients can be treated by gene replacement therapy. One or more copies of a normal HBMYCNG gene or a portion of the gene that directs the production of a normal HBMYCNG gene protein with HBMYCNG gene function, can be inserted into cells, via, for example a delivery complex as described *supra*.

Such gene replacement techniques can be accomplished either *in vivo* or *in vitro*. Techniques which select for expression within the cell type of interest are preferred. For *in vivo* applications, such techniques can, for example, include appropriate local administration of HBMYCNG gene sequences.

Additional methods which may be utilized to increase the overall level of HBMYCNG activity include the introduction of appropriate HBMYCNG gene-expressing cells, preferably autologous cells, into a patient at positions and in numbers which are sufficient to ameliorate the symptoms of the ion/cation-related disorder. Such cells may be either recombinant or non-recombinant. Among the cells which can be



administered to increase the overall level of HBMYCNG  
 5 gene expression in a patient are normal cells, which  
 express the HBMYCNG gene. The cells can be administered  
 at the anatomical site of expression, or as part of a  
 tissue graft located at a different site in the body.  
 Such cell-based gene therapy techniques are well known to  
 10 those skilled in the art (see, e.g., Anderson, et al.,  
 United States Patent No. 5,399,349; Mulligan and Wilson,  
 United States Patent No. 5,460,959).

HBMYCNG gene sequences can also be introduced into  
 autologous cells *in vitro*. These cells expressing the  
 15 HBMYCNG gene sequence can then be reintroduced,  
 preferably by intravenous administration, into the  
 patient until the disorder is treated and symptoms of the  
 disorder are ameliorated.

#### 20 5.4.3.3. Additional Modulatory Techniques

The present invention also includes modulatory  
 techniques which, depending on the specific application  
 for which they are utilized, can yield either an increase  
 or a decrease in HBMYCNG activity levels leading to the  
 25 amelioration of ion/cation-related disorders such as  
 those described above.

Antibodies exhibiting modulatory capability can be  
 utilized according to the methods of this invention to  
 treat the ion/cation-related disorders. Depending on the  
 30 specific antibody, the modulatory effect can be an  
 increase or decrease in HBMYCNG activity. Such antibodies  
 can be generated using standard techniques described in  
 Section 5.3, *supra*, against full length wild type or  
 mutant HBMYCNG proteins, or against peptides  
 35 corresponding to portions of the proteins, as wells as  
 against extracellular domains of the HBMYCNG polypeptide  
 or HBMYCNG epitopes within the water soluble fusion

protein mimic of the HMBYCNG disclosed above. The  
 5 antibodies include but are not limited to polyclonal,  
 monoclonal, Fab fragments, single chain antibodies,  
 chimeric antibodies, etc.

Lipofectin or liposomes can be used to deliver the  
 antibody or a fragment of the Fab region which binds to  
 10 the HMBYCNG gene product epitope to cells expressing the  
 gene product. Where fragments of the antibody are used,  
 the smallest inhibitory fragment which binds to the  
 HMBYCNG protein's binding domain is preferred. For  
 example, peptides having an amino acid sequence  
 15 corresponding to the domain of the variable region of the  
 antibody that binds to the HMBYCNG protein can be used.  
 Such peptides can be synthesized chemically or produced  
 via recombinant DNA technology using methods well known  
 in the art (e.g., see Creighton, 1983, *supra* and Sambrook  
 20 et al., 1989, *supra*). Alternatively, single chain  
 antibodies, such as neutralizing antibodies, which bind  
 to intracellular epitopes can also be administered. Such  
 single chain antibodies can be administered, for example,  
 by expressing nucleotide sequences encoding single-chain  
 25 antibodies within the target cell population by  
 utilizing, for example, techniques such as those  
 described in Marasco et al., 1993, Proc. Natl. Acad. Sci.  
 USA 90:7889-7893.

### 30 5.5. Pharmaceutical Preparations And Methods of

#### Administration

The compounds, e.g., nucleic acid sequences,  
 polypeptides, peptides, and recombinant cells, described  
*supra* can be administered to a patient at therapeutically  
 35 effective doses to treat or ameliorate ion/cation-related  
 disorders. A therapeutically effective dose refers to  
 that amount of a compound or cell population sufficient

to result in amelioration of the disorder symptoms, or  
5 alternatively, to that amount of a nucleic acid sequence  
sufficient to express a concentration of HBMYCNG gene  
product which results in the amelioration of the disorder  
symptoms.

Toxicity and therapeutic efficacy of compounds can  
10 be determined by standard pharmaceutical procedures in  
cell cultures or experimental animals, e.g., for  
determining the LD50 (the dose lethal to 50% of the  
population) and the ED50 (the dose therapeutically  
effective in 50% of the population). The dose ratio  
15 between toxic and therapeutic effects is the therapeutic  
index and it can be expressed as the ratio LD50/ED50.  
Compounds which exhibit large therapeutic indices are  
preferred. While compounds that exhibit toxic side  
effects can be used, care should be taken to design a  
20 delivery system that targets such compounds to the site  
of affected tissue in order to minimize potential damage  
to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and  
animal studies can be used in formulating a range of  
25 dosage for use in humans. The dosage of such compounds  
lies preferably within a range of circulating  
concentrations that include the ED50 with little or no  
toxicity. The dosage can vary within this range depending  
upon the dosage form employed and the route of  
30 administration utilized. For any compound used in the  
methods of the invention, the therapeutically effective  
dose can be estimated initially from cell culture assays.  
A dose can be formulated in animal models to achieve a  
circulating plasma concentration range that includes the  
35 IC50 (i.e., the concentration of the test compound which  
achieves a half-maximal inhibition of symptoms) as  
determined in cell culture. Such information can be used

to more accurately determine useful doses in humans.

- 5 Levels in plasma can be measured, for example, by high performance liquid chromatography.

Pharmaceutical compositions for use in accordance with the present invention can be formulated in conventional manner using one or more physiologically acceptable carriers or excipients.

- Thus, the compounds and their physiologically acceptable salts and solvents can be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, 15 parenteral or rectal administration.

For oral administration, the pharmaceutical compositions can take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinised maize starch, 20 polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets can be coated by methods well known in the art. Liquid preparations for oral administration can take the form of, for example, solutions, syrups or suspensions, or they can be 30 presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations can be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or 35 hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, ethyl alcohol or fractionated vegetable

oils); and preservatives (e.g., methyl or  
 5 propyl-p-hydroxybenzoates or sorbic acid). The  
 preparations can also contain buffer salts, flavoring,  
 coloring and sweetening agents as appropriate.

Preparations for oral administration can be suitably  
 formulated to give controlled release of the active  
 10 compound.

For buccal administration the compositions can take  
 the form of tablets or lozenges formulated in  
 conventional manner.

For administration by inhalation, the compounds for  
 15 use according to the present invention are conveniently  
 delivered in the form of an aerosol spray presentation  
 from pressurized packs or a nebulizer, with the use of a  
 suitable propellant, e.g., dichlorodifluoromethane,  
 trichlorofluoromethane, dichlorotetrafluoroethane, carbon  
 20 dioxide or other suitable gas. In the case of a  
 pressurized aerosol the dosage unit can be determined by  
 providing a valve to deliver a metered amount. Capsules  
 and cartridges of e.g. gelatin for use in an inhaler or  
 insufflator can be formulated containing a powder mix of  
 25 the compound and a suitable powder base such as lactose  
 or starch.

The compounds can be formulated for parenteral  
 administration (i.e., intravenous or intramuscular) by  
 injection, via, for example, bolus injection or  
 30 continuous infusion. Formulations for injection can be  
 presented in unit dosage form, e.g., in ampoules or in  
 multi-dose containers, with an added preservative. The  
 compositions can take such forms as suspensions,  
 solutions or emulsions in oily or aqueous vehicles, and  
 35 can contain formulatory agents such as suspending,  
 stabilizing and/or dispersing agents. Alternatively, the  
 active ingredient can be in powder form for constitution

with a suitable vehicle, e.g., sterile pyrogen-free  
5 water, before use. It is preferred that  
HBMYCNG-expressing cells be introduced into patients via  
intravenous administration.

The compounds can also be formulated in rectal  
compositions such as suppositories or retention enemas,  
10 e.g., containing conventional suppository bases such as  
cocoa butter or other glycerides.

In addition to the formulations described  
previously, the compounds can also be formulated as a  
depot preparation. Such long acting formulations can be  
15 administered by implantation (for example subcutaneously  
or intramuscularly) or by intramuscular injection. Thus,  
for example, the compounds can be formulated with  
suitable polymeric or hydrophobic materials (for example  
as an emulsion in an acceptable oil) or ion exchange  
20 resins, or as sparingly soluble derivatives, for example,  
as a sparingly soluble salt.

The compositions can, if desired, be presented in a  
pack or dispenser device which can contain one or more  
unit dosage forms containing the active ingredient. The  
25 pack can for example comprise metal or plastic foil, such  
as a blister pack. The pack or dispenser device can be  
accompanied by instructions for administration.

#### 6. Example: Identification of Two Novel HBMYCNG Genes and Their Encoded Proteins

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30 The section below describes the identification of a  
novel human CNG gene sequence encoding the full-length,  
novel human ion channel, HBMYCNG.

##### 6.1. Cloning of Novel HBMYCNG DNA Sequences

35 In general all routine molecular biology procedures  
followed standard protocols or relied on widely available  
commercial kits and reagents. All sequencing was done

with an ABI 373 automated sequencer using commercial  
5 dye-terminator chemistry.

Cyclic nucleotide gated channel sequences from rat,  
mouse and chicken were used as sequence probes in a  
homology search (gapped BLAST) of public domain expressed  
sequence tag (EST) and human genomic databases. The top  
10 EST and genomic hits from the BLAST search, (*i. e.* those  
BLAST hits whose Expectation values were less than 0.001  
were selected as potential hits and selected for  
subsequent analysis) were used as probes in a second  
homology search against the non-redundant protein and  
15 patent sequence databases. The results of the second  
search revealed putative genomic exons which could encode  
a novel CNG ion channel, within Bacterial Artificial  
Chromosome (BAC), Accession No. AF002992.

The cDNA complete coding sequence of the HBMYCNG  
20 gene was cloned as follows. Using the predict full length  
sequence The following PCR Primers were designed.

	HuCNG2-s	GCTCTAGATGTACATGGAGGATGACCGAAA	Xba 1 site
25	HuCNG2-1 a	CAGCCAACGCAGTCTGTACTCT	no sites, use nested primer 2
30	HuCNG2-2 a	CGGGATCCGAGGCGGAATCTTGGATGTTT	BamH1 site

Using huCNG2-s and huCNG2-1a, PCR was carried out on  
brain first strand cDNA made by standard techniques. To  
increase the specificity of the amplification, a 1  
35 microliter aliquot was removed after the PCR reaction was  
complete and re-amplified using huCNG2-s and huCNG2-2a.  
The PCR reaction was passed over a s-400 spun-column

(Amersham Pharmacia Biotech, Piscataway, NJ) to remove  
 5 excess PCR primers and DNA was digested with the  
 restriction endonucleases Xba I and Bam HI. This reaction  
 was extracted with phenol:chloroform and the aqueous  
 layer precipitated with 100 % ethanol and 0.3 M Sodium  
 Acetate. The precipitated DNA was run on an 0.8% agarose  
 10 gel and the DNA band purified using a QIAquick Gel  
 extraction kit (Qiagen, Valencia CA). The resulting DNA  
 was ligated to pBS-SK digested with Xba I and BamHI  
 (Stratagene, La Jolla, CA) and introduced into E. coli  
 strain DH10B using standard techniques. Positive clones  
 15 were identified by PCR, using the same primers used for  
 cloning, and several clones were sequenced using the PCR  
 primers as well as with internal primers designed from  
 the predicted gene sequence.

20	CNG2-3s	AGAGCCTGCTTCAGTGA	17	Sequencing primer
	CNG2-3a	TCACTGAAGCAGGCTCT	17	Sequencing primer
	CNG2-4s	TTACTGGTCCACACTGA	17	Sequencing primer
25	CNG2-4a	TCAGTGTGGACCAGTAA	17	Sequencing primer
	CNG2-5s	ACGCACAGCTAATATCCGCA	20	Sequencing primer
	CNG2-5a	TGCGGATATTAGCTGTGCGT	20	Sequencing primer

30 The resulting sequence was compared to the predicted  
 sequence for completeness.

The DNA sequence for HBMYCNG is depicted in FIG. 1.  
 The derived protein, i.e., the full-length amino acid  
 35 sequence encoded by the HBMYCNG gene is depicted in FIG.  
 2. Analysis of the amino acid sequence of Fig. 2 for the  
 detection of transmembrane segments was performed using



the computer program TMPRED and transmembrane prediction  
 5 information from related proteins. Putative transmembrane  
 segments are depicted in bold in Fig. 3, while the  
 predicted ion pore, located between the fifth and sixth  
 transmembrane, counting from the amino-terminus of the  
 protein, is underlined.

10 The complete sequence for HBMYCNG can be identified  
 in a set of sequences from a large genomic fragment  
 (AF002992) reported as part of the human genome  
 sequencing project. The complete cDNA nucleotide sequence  
 encoding the HBMYCNG polypeptide described herein was  
 15 only partially identified in the annotations to the  
 AF002992 BAC sequence.

## 6.2. Calcium Flux Assays Using the HBMYCNG Gene

$\text{Ca}^{2+}$ -flux assays are performed to determine the  
 20 effect on HBMYCNG of various ligands known to affect  
 cation channel proteins. More specifically,  $\text{Ca}^{2+}$  uptake is  
 measured in transiently transfected CHO cells, i.e.,  
 transfected with the HBMYCNG nucleic acid molecules of  
 the invention, using the  $\text{Ca}^{2+}$ -sensitive dye Fluo-4  
 25 (Molecular Probes) in a Molecular Devices Fluorometric  
 Imaging Plate Reader (FLIPR). Cells are loaded with the  
 dye for 30-90 minutes prior to the experiment in the  
 presence of sulfinpyrazone. Test reagents are added, and  
 $\text{Ca}^{2+}$  uptake measured over a three minute period.

30  $\text{Ca}^{2+}$ -flux assays may also be performed for the  
 detection and evaluation of compounds that modulate the  
 activity of G-protein coupled receptors. In such assays,  
 cells expressing a G-protein coupled receptor of interest  
 are loaded with the dye for 30-90 minutes prior to the  
 35 experiment in the presence of sulfinpyrazone. Test  
 reagents, which include test compounds, which may be  
 agonists or antagonists of the G-protein coupled receptor

are added, and  $\text{Ca}^{2+}$  uptake, reflecting the intracellular cyclic nucleotide concentration, is measured over a three minute period. In addition, these same assay techniques can be applied to other cations that enter cells through CNG channels, using appropriate dyes and incubations.

### 10 6.3. Expression Profile of HBMYCNG

The expression profile of HBMYCNG in various tissues was determined by measuring the relative abundance of HBMYCNG RNA in those tissues using quantitative PCR analyses.

15

#### Methods

Total RNA from tissues was isolated using the TriZol protocol (Invitrogen, Carlsbad, CA) and quantified by determining absorbance at 260nm. An assessment of the 18S and 28S ribosomal RNA bands was made by denaturing gel electrophoresis to determine RNA integrity.

The specific sequence to be measured was aligned with related genes found in GenBank to identify regions of significant sequence divergence to maximize primer and probe specificity. Gene-specific primers and probes were designed using ABI Primer Express software (Applied Biosystems, Foster City, CA) and used to amplify small amplicons (150 base pairs or less) to maximize the likelihood that the primers would function at 100% efficiency. The primer and probe sequences were searched against Public Genbank databases to ensure target specificity. Primers and probes were obtained from ABI.

For HBMYCNG the primer and probe sequences used were:

35 Forward Primer 5'-TCAGAGAATGGGCCAACAAGA-3'  
Reverse Primer 5'-CGAAAACGCTCGAGGAATGA-3'  
Probe CAGGCCTAGGTTCTCTCTCGGAA

## 5        DNA contamination

To assess the level of contaminating genomic DNA in the RNA, the RNA was divided into 2 aliquots and one half was treated with Rnase-free Dnase (Invitrogen, Carlsbad, CA). RNA from both the Dnase-treated and non-treated

10 samples were then subjected to reverse transcription reactions with (RT+) and without (RT-) the presence of reverse transcriptase. TaqMan™ assays were carried out with the gene-specific primers (see below) and the contribution of genomic DNA to the signal detected was

15 evaluated by comparing the threshold cycles obtained with the RT+/RT- non-Dnase treated RNA to that on the RT+/RT- Dnase treated RNA. For the RNA samples used for the determination of relative expression levels, the amount of signal contributed by genomic DNA in the Dnased RT-

20 RNA was less than 10% of that obtained with Dnased RT+ RNA.

## Reverse Transcription reaction and Sequence Detection

25        100ng of Dnase-treated total RNA was annealed to 2.5 mM of the gene-specific reverse primer in the presence of 5.5 mM MgCl<sub>2</sub> by heating the sample to 72°C for 2 min and then cooling to 55° C for 30 min. 1.25 U/ml of MuL<sub>v</sub> reverse transcriptase and 500mM of each dNTP were then

30 added to the reaction and the sample was incubated at 37° C for 30 min. The sample was then heated to 90°C for 5 min to denature enzyme.

Quantitative sequence detection was carried out on a ABI PRISM 7700 by adding the following components to the

35 reverse transcribed reaction: forward and reverse primers (each to a concentration of 2.5mM), all four dNTPs (500mM each), buffer and 5U AmpliTaq Gold™. The PCR reaction is

then held at 94°C for 12 min, followed by 40 amplification  
5 cycles of 94° C for 15 sec and 60° C for 30 sec.

#### Data Analysis

The threshold cycle (Ct) of the lowest expressing  
tissue (the highest Ct value) was used as the baseline of  
expression and all other tissues were expressed as the  
10 relative abundance to that tissue by calculating the  
difference in Ct value between the baseline and the other  
tissues and using it as the exponent in  $2^{(\Delta Ct)}$ . The  
threshold cycles for testis, raphe nucleus, and pineal  
gland were 32, 36.5, and 37.5, respectively, indicating  
15 that the number of copies of HBMYCNG mRNA in these  
samples was very low.

#### Results

The data obtained indicated that the HBMYCNG gene is  
20 expressed only in certain tissues and only at very low  
levels in those tissues. More specifically, expression of  
the HBMYCNG gene is 250-fold greater in testis, 10-fold  
greater in the raphe nucleus of the brain, and 5-fold  
greater in the pineal gland than in control tissues.

25

#### 6.4. HBMYCNG Fusion Proteins

Chimeric proteins comprising all or a portion of the  
HBMYCNG protein, as depicted in FIG. 2, fused to all or a  
portion of a heterologous protein, are provided using  
30 recombinant DNA methods and reagents well known in the  
art. In specific embodiments, one or more portions of the  
HBMYCNG protein are fused to a portion of an  
immunoglobulin protein, and, more particularly, to a  
portion of a human IgG comprising the hinge, CH2, and CH3  
35 regions thereof.

Such portions of the HBMYCNG protein can include,  
but are not limited to, one more of the extracellular

- domains of the HBMYCNG protein, comprising,
- 5 approximately, amino acid residues 161 to 173, amino acid residues 237 to 274, and amino acid residues 370 to 453 of SEQ ID No.: 2. In other embodiments, the portion of the HBMYCNG protein incorporated into a fusion includes all or a portion of the amino terminal domain of the
- 10 HBMYCNG protein, comprising, approximately, amino acid residues 1 or 2 to residue 140 SEQ ID No.: 2, or of the carboxy-terminal domain of the HBMYCNG protein, comprising, approximately amino acid residues 474 to 644 of SEQ ID No.: 2.
- 15 DNA encoding the desired portion of the HBMYCNG protein can be isolated by PCR amplification of appropriate sequences, using, for example, cDNA as template, preferably cloned cDNA comprising the nucleotide sequence of SEQ ID NO.: 1, and appropriate
- 20 upstream and downstream primers. The design, synthesis, and use of such primers are well known in the art and will include, as needed or desired, appropriate recognition sequences for one or more restriction enzymes to enable directional, in-frame cloning of a DNA fragment
- 25 encoding a particular portion of the HBMYCNG protein into an expression vector in operable association with appropriate genetic expression and regulatory elements and with a second DNA sequence encoding the protein or portion thereof to which the HBMYCNG protein portion is
- 30 to be fused. Examples of systems useful for the expression of such fusion proteins, in which the HBMYCNG protein portion may be positioned at either the amino-terminus, carboxyl-terminus or within a chimeric fusion protein, are disclosed *supra*.
- 35 HBMYCNG-immunoglobulin C gamma (IgC $\gamma$ ) fusion proteins are prepared as described by Linsley et al., in J. Exp. Med. 173:721-730 (1991), which is hereby incorporated by

reference in its entirety, incorporated by reference  
5 herein. DNA encoding amino acid sequences corresponding  
to the desired portion of the HBMYCNG protein are joined  
to DNA encoding amino acid sequences corresponding to the  
hinge, CH2 and CH3 regions of human IgC $\gamma$ 1. This is  
accomplished using PCR amplification to generate DNA  
10 fragments encoding appropriate portions of the HBMYCNG  
and IgC $\gamma$  proteins. PCR reactions (0.1 ml final volume)  
are run in Taq polymerase buffer(Stratagene, La Jolla,  
Calif.), containing 20 $\mu$  moles each of dNTP; 50-100 pmoles  
of the appropriate primers; template (1 ng plasmid or  
15 cDNA synthesized as described by Kawasaki in PCR  
Protocols, Academic Press, pp. 21-27 (1990),incorporated  
by reference herein); and Taq polymerase (Stratagene).  
Reactions are run on a thermocycler (Perkin Elmer Corp.,  
Norwalk, Conn.)for 16-30 cycles (a typical cycle consists  
20 of steps of 1 min at 94 °C., 1-2 min at 50 °C. and 1-3 min  
at 72 °C). Products of the PCR reactions are cleaved with  
appropriate restriction endonucleases at sites introduced  
in the PCR primers, and then are gel purified.

The 3' portion of the fusion constructs  
25 corresponding to human IgC $\gamma$ 1 sequences is was made by a  
coupled reverse transcriptase (from Avian myeloblastosis  
virus; Life Sciences Associates, Bayport, N.Y.)--PCR  
reaction using RNA from a myeloma cell line producing  
human-mouse chimeric mAb L6 (available from Dr. P. Fell  
30 and M. Gayle, Bristol-Myers Squibb Company,  
Pharmaceutical Research Institute, Seattle, Wash.) as  
template. Appropriate upstream and downstream  
oligonucleotide, such as those described in U.S. Patent  
No. 6,090,914,which is hereby incorporated by reference  
35 in its entirety, are used to amplify and isolate the  
desired IgC $\gamma$  coding region.

Reaction products are cleaved with appropriate  
 5 restriction endonucleases and gel purified. Final  
 constructs are assembled by ligating the endonucleases  
 cleaved fragments containing HBMYCNG sequence together  
 with a cleaved fragment containing IgC $\gamma$ 1 sequences into  
 an expression vector such as CDMB, as described in U.S.  
 10 Patent No. 6,090,917. Ligation products are transformed  
 into MC1061/p3 *E. coli* cells and colonies are screened  
 for the appropriate plasmids. Sequences of the resulting  
 constructs are confirmed by DNA sequencing. In a  
 preferred embodiment the HBMYCNG portion coding sequence  
 15 is fused in this manner to DNA encoding amino acids  
 corresponding to the IgC $\gamma$ 1 hinge region.

#### Cell Culture and Transfections

COS (monkey kidney cells) are transfected with  
 20 expression these chimeric fusion proteins using a  
 modification of the protocol of Seed and Aruffo (Proc.  
 Natl. Acad. Sci. 84:3365 (1987)), incorporated by  
 reference herein. Cells are seeded at  $10^6$  per 10 cm  
 diameter culture dish 18-24 h before transfection.  
 25 Plasmid DNA is added (approximately 15  $\mu$ g/dish) in a  
 volume of 5 mls of serum-free DMEM containing 0.1 mM  
 chloroquine and 600  $\mu$ g/ml DEAE Dextran, and cells are  
 incubated for 3-3.5 h at 37 °C. Transfected cells are then  
 briefly treated (approximately 2 min) with 10% dimethyl  
 30 sulfoxide in PBS and incubated at 37 °C for 16-24 h in  
 DMEM containing 10% FCS. At 24 h after transfection,  
 culture medium is removed and replaced with serum-free  
 DMEM (6 ml/dish). Incubation is continued for 3 days at  
 37 °C, at which time the spent medium is collected and  
 35 fresh serum-free medium is added. After an additional 3  
 days at 37 °C, the spent medium is again collected and  
 cells are discarded. CHO cells expressing HBMYCNG-IgC $\gamma$

fusion proteins are isolated as described by Linsley et al., (1991) supra, as follows: stable transfectants expressing the desired fusion protein are isolated following cotransfection of dihydrofolate reductase-deficient Chinese hamster ovary (dhfr- CHO) cells with a mixture of the appropriate expression plasmid and the selectable marker, pSV2dhfr (Linsley et al., Proc. Natl. Acad. Sci. USA 87:5031 (1990)), incorporated by reference herein. Transfectants are then grown in increasing concentrations of methotrexate to a final level of 1  $\mu$ M and were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 0.2 mM proline and 1  $\mu$ M methotrexate. CHO lines expressing high levels of the desired fusion proteins are isolated by multiple rounds of fluorescence-activated cell sorting following indirect immunostaining with an appropriate labeled anti-HBMYCNG mAb.

#### Purification of Ig Fusion Proteins

The first, second and third collections of spent serum-free culture media from transfected COS cells are used as sources for the purification of Ig fusion proteins. After removal of cellular debris by low speed centrifugation, medium is applied to a column (approximately 200-400 ml medium/ml packed bed volume) of immobilized protein A (Repligen Corp., Cambridge, Mass.) equilibrated with 0.05 M sodium citrate, pH 8.0. After application of the medium, the column is washed with 1 M potassium phosphate, pH 8, and bound protein is eluted with 0.05 M sodium citrate, pH 3. Fractions were collected and immediately neutralized by addition of 1/10 volume of 2 M Tris, pH 8. Fractions containing the peak of  $A_{280}$  absorbing material are pooled and dialyzed against PBS before use.



## 5 6.5 Preparation of Antibodies Directed Against HBMYCNG Epitopes

Antibodies of the present invention can be prepared by a variety of methods. In one method, purified HBMYCNG antigen or cells expressing purified HBMYCNG antigen are administered to an animal to induce the production of sera containing polyclonal antibodies. In a preferred method, a preparation of HBMYCNG antigen is purified to homogeneity before being administered to an animal to provide polyclonal antisera of greater specific activity. In certain embodiments, soluble portions of the HBMYCNG protein are used as the immunogen for generation of antibodies. Such soluble portions include, but are not limited to extracellular domains of the HBMYCNG protein which comprise, approximately residues 161 to 173, amino acid residues 237 to 274, and amino acid residues 370 to 453 of SEQ ID No.: 2. In other embodiments, a soluble portion of the HBMYCNG protein used as an immunogen may include all or a portion of the amino terminal domain of the HBMYCNG protein, comprising, approximately, amino acid residues 1 or 2 to residue 140 SEQ ID No.: 2, or all or a portion of the carboxy-terminal domain of the HBMYCNG protein, comprising, approximately amino acid residues 474 to 644 of SEQ ID No.: 2. In other embodiments the immunogen administered to the animal may be a chimeric protein or peptide comprising a portion, particularly a soluble portion, of the HBMYCNG protein fused to a protein, polypeptide, or peptide carrier. Such fusions may be constructed by genetic engineering or may be formed by chemical conjugation of the HBMYCNG protein or peptide to a suitable carrier protein or peptide using methods well known in the art.

Monoclonal antibodies specific for the HBMYCNG  
 5 protein, or a portion thereof, are prepared using  
 hybridoma technology. (Kohler et al., Nature 256:495  
 (1975); Kohler et al., Eur. J. Immunol. 6:511 (1976);  
 Kohler et al., Eur. J. Immunol. 6:292 (1976); Hammerling  
 et al., in: Monoclonal Antibodies and T-Cell Hybridomas,  
 10 Elsevier, N.Y., pp. 563-681 (1981)).

An animal, preferably a mouse, is immunized with the  
 HBMYCNG protein or a portion thereof and then splenocytes  
 of the immunized mice are extracted and fused with a  
 suitable myeloma cell line. Any suitable myeloma cell  
 15 line may be employed in accordance with the present  
 invention; however, it is preferable to employ the parent  
 myeloma cell line (SP20), available from the ATCC. After  
 fusion, the resulting hybridoma cells are selectively  
 maintained in HAT medium, and then cloned by limiting  
 20 dilution as described by Wands et al. (Gastroenterology  
 80:225-232 (1981). Hybridoma cells obtained through such  
 a selection are then assayed to identify clones which  
 secrete antibodies capable of binding the HBMYCNG  
 polypeptide or portion thereof.

For *in vivo* use of antibodies in humans, an antibody  
 is "humanized". Such antibodies can be produced using  
 genetic constructs derived from hybridoma cells producing  
 the monoclonal antibodies described above. Methods for  
 producing chimeric and humanized antibodies are known in  
 30 the art as disclosed above. (See also, Morrison, Science  
 229:1202 (1985); Oi et al., BioTechniques 4:214 (1986);  
 Cabilly et al., U.S. Patent No. 4,816,567; Taniguchi et  
 al., EP 171496; Morrison et al., EP 173494; Neuberger et  
 al., WO 8601533; Robinson et al., WO 8702671; Boulianne  
 35 et al., Nature 312:643 (1984); Neuberger et al., Nature  
 314:268 (1985).)

Isolation Of Antibody Fragments Directed Against the  
HBMYCNG Protein From a Library Of scFvs

- 5 Naturally occurring V-genes isolated from human peripheral blood lymphocytes (PBLs) are constructed into a library of antibody fragments which contain reactivities against the HBMYCNG protein to which the
- 10 donor may or may not have been exposed (see e.g. Marks et al. J. Mol. Bio. 222(3): 581-97 (1991), and U.S. Patent 5,885,793, each of which is incorporated herein by reference in its entirety).

- A library of scFvs is constructed from the RNA of
- 15 human PBLs as described in PCT publication WO 92/01047, which is hereby incorporated by reference in its entirety. To rescue phage displaying antibody fragments, approximately  $10^9$  *E. coli* harboring the phagemid are used to inoculate 50 ml of 2xTY containing 1% glucose and 100
- 20  $\mu$ g/ml of ampicillin (2xTY-AMP-GLU) and grown to an O.D. of 0.8 with shaking. Five ml of this culture are used to inoculate 50 ml of 2xTY-AMP-GLU,  $2 \times 10^8$  transforming units (TU) of M13  $\Delta$  gene III helper phage (PCT publication WO 92/01047) are added and the culture
- 25 incubated at 37 °C for 45 minutes without shaking and then at 37 °C for 45 minutes with shaking. The culture is centrifuged at 4000 r.p.m. for 10 min. and the pellet resuspended in 2 liters of 2xTY containing 100  $\mu$ g/ml ampicillin and 50 ug/ml kanamycin and grown overnight.
- 30 Phage are prepared as described in PCT publication WO 92/01047.

- M13  $\Delta$  gene III is prepared as follows: M13  $\Delta$  gene III helper phage does not encode gene III protein, hence the phage(mid) displaying antibody fragments have a
- 35 greater avidity of binding to antigen. Infectious M13  $\Delta$  gene III particles are prepared by growing the helper phage in cells harboring a pUC19 derivative supplying the

wild type gene III protein during phage morphogenesis.

- 5 The culture is incubated for 1 hour at 37 °C without shaking and then for a further hour at 37 °C with shaking. Cells are collected by centrifugation, resuspended in 300 ml 2xTY broth containing 100 µg ampicillin/ml and 25 µg kanamycin/ml (2xTY-AMP-KAN) and grown overnight, shaking
- 10 at 37 °C. Phage particles are purified and concentrated from the culture medium by two PEG-precipitations, resuspended in 2 ml PBS and passed through a 0.45 µm filter (Minisart NML; Sartorius) to give a final concentration of approximately  $10^{13}$  transducing units/ml
- 15 (ampicillin-resistant clones).

- Panning of the Library. Immunotubes (Nunc) are coated overnight in PBS with 4 ml of either 100 µg/ml or 10 µg/ml of BMVNC protein or portion thereof and then blocked with 2% Marvel-PBS for 2 hours at 37 °C and then
- 20 washed 3 times in PBS. Approximately  $10^{13}$  TU of phage is applied to the tube and incubated for 30 minutes at room temperature tumbling on an over and under turntable and then left to stand for another 1.5 hours. Tubes are washed 10 times with PBS 0.1% Tween-20 and 10 times with
  - 25 PBS. Phage are eluted by adding 1 ml of 100 mM triethylamine and rotating 15 minutes on an under and over turntable after which the solution is immediately neutralized with 0.5 ml of 1.0M Tris-HCl, pH 7.4. Phage are then used to infect 10 ml of mid-log *E. coli* TG1 by
  - 30 incubating eluted phage with bacteria for 30 minutes at 37 °C. The *E. coli* are then plated on TYE plates containing 1% glucose and 100 µg/ml ampicillin. The resulting bacterial library is then rescued with  $\Delta$  gene III helper phage as described above to prepare phage for
  - 35 a subsequent round of selection. This process is then repeated for a total of 4 rounds of affinity purification

- with tube-washing increased to 20 times with PBS, 0.1% Tween-20 and 20 times with PBS for rounds 3 and 4.

Eluted phage from the 3rd and 4th rounds of selection are used to infect *E. coli* HB 2151 and soluble scFv is produced (Marks et al. J. Mol. Bio. 222(3): 581-97 (1991)) from single colonies for assay. ELISAs are performed with microtitre plates coated with either 10 pg/ml of HBMYCNG protein or a portion thereof in 50 mM bicarbonate pH 9.6. Clones positive in ELISA are further characterized by PCR fingerprinting (see, e.g., PCT publication WO 92/01047) and then by sequencing.

15

6.5 Method of Creating N- and C-terminal Deletion Mutants Corresponding to the HBMYCNG Polypeptide of the Present Invention.

- As described elsewhere herein, the present invention encompasses the creation of N- and C-terminal deletion mutants, in addition to any combination of N- and C-terminal deletions thereof, corresponding to the HBMYCNG polypeptide of the present invention. A number of methods are available to one skilled in the art for creating such mutants. Such methods may include a combination of PCR amplification and gene cloning methodology. Although one of skill in the art of molecular biology, through the use of the teachings provided or referenced herein, and/or otherwise known in the art as standard methods, could readily create each deletion mutant of the present invention, exemplary methods are described below.

Briefly, using the isolated cDNA clone encoding the full-length HBMYCNG polypeptide sequence (as described herein, for example), appropriate primers of about 15-25 nucleotides derived from the desired 5' and 3' positions of SEQ ID NO:1 may be designed to PCR amplify, and subsequently clone, the intended N- and/or C-terminal deletion mutant. Such primers could comprise, for example,

an initiation and stop codon for the 5' and 3' primer,  
 5 respectively. Such primers may also comprise restriction  
 sites to facilitate cloning of the deletion mutant post  
 amplification. Moreover, the primers may comprise  
 additional sequences, such as, for example, flag-tag  
 sequences, kozac sequences, or other sequences discussed  
 10 and/or referenced herein.

For example, in the case of the Y140 to P664 N-  
 terminal deletion mutant, the following primers could be  
 used to amplify a cDNA fragment corresponding to this  
 deletion mutant:

15

5' Prime r	5'-GCAGCA <u>GCGGCCGC</u> TACTACTGCTGGCTATTTGTCATTG-3' (SEQ ID NO:19)  <b><i>NotI</i></b>
3' Prime r	5'- GCAGCA <u>GTCGAC</u> TGGCTCGTCAGCAGCAGCCAGCTC-3' (SEQ ID NO:20)  <b><i>Sall</i></b>

20

For example, in the case of the M1 to F475 C-terminal  
 deletion mutant, the following primers could be used to  
 25 amplify a cDNA fragment corresponding to this deletion  
 mutant:

30

5' Prime r	5'- GCAGCA <u>GCGGCCGC</u> ATGACCGAAAAACCAATGGTGTG-3' (SEQ ID NO:21)  <b><i>NotI</i></b>
3' Prime r	5'- GCAGCA <u>GTCGAC</u> GAAGACCTGAGGACGGAGTTTCAG-3' (SEQ ID NO:22)  <b><i>Sall</i></b>

Representative PCR amplification conditions are  
 35 provided below, although the skilled artisan would  
 appreciate that other conditions may be required for  
 efficient amplification. A 100 ul PCR reaction mixture may  
 be prepared using 10ng of the template DNA (cDNA clone of

HBMYCNG), 200 uM 4dNTPs, 1uM primers, 0.25U Taq DNA  
 5 polymerase (PE), and standard Taq DNA polymerase buffer.  
 Typical PCR cycling condition are as follows:

20-25 cycles: 45 sec, 93 degrees  
                   2 min, 50 degrees  
 10                2 min, 72 degrees  
                   1 cycle: 10 min, 72 degrees

After the final extension step of PCR, 5U Klenow  
 Fragment may be added and incubated for 15 min at 30  
 15 degrees.

Upon digestion of the fragment with the NotI and SalI  
 restriction enzymes, the fragment could be cloned into an  
 appropriate expression and/or cloning vector which has been  
 similarly digested (e.g., pSport1, among others). . The  
 20 skilled artisan would appreciate that other plasmids could  
 be equally substituted, and may be desirable in certain  
 circumstances. The digested fragment and vector are then  
 ligated using a DNA ligase, and then used to transform  
 competent E.coli cells using methods provided herein and/or  
 25 otherwise known in the art.

The 5' primer sequence for amplifying any additional  
 N-terminal deletion mutants may be determined by reference  
 to the following formula:

$$(S+(X * 3)) \text{ to } ((S+(X * 3))+25),$$
 wherein 'S' is equal  
 30 to the nucleotide position of the initiating start codon of  
 the HBMYCNG gene (SEQ ID NO:1), and 'X' is equal to the  
 most N-terminal amino acid of the intended N-terminal  
 deletion mutant. The first term will provide the start 5'  
 nucleotide position of the 5' primer, while the second term  
 35 will provide the end 3' nucleotide position of the 5'  
 primer corresponding to sense strand of SEQ ID NO:1. Once  
 the corresponding nucleotide positions of the primer are  
 determined, the final nucleotide sequence may be created by

the addition of applicable restriction site sequences to the 5' end of the sequence, for example. As referenced herein, the addition of other sequences to the 5' primer may be desired in certain circumstances (e.g., kozak sequences, etc.).

The 3' primer sequence for amplifying any additional N-terminal deletion mutants may be determined by reference to the following formula:

$$(S+(X * 3)) \text{ to } ((S+(X * 3))-25),$$
 wherein 'S' is equal to the nucleotide position of the initiating start codon of the HBMYCNG gene (SEQ ID NO:1), and 'X' is equal to the most C-terminal amino acid of the intended N-terminal deletion mutant. The first term will provide the start 5' nucleotide position of the 3' primer, while the second term will provide the end 3' nucleotide position of the 3' primer corresponding to the anti-sense strand of SEQ ID NO:1. Once the corresponding nucleotide positions of the primer are determined, the final nucleotide sequence may be created by the addition of applicable restriction site sequences to the 5' end of the sequence, for example. As referenced herein, the addition of other sequences to the 3' primer may be desired in certain circumstances (e.g., stop codon sequences, etc.). The skilled artisan would appreciate that modifications of the above nucleotide positions may be necessary for optimizing PCR amplification.

The same general formulas provided above may be used in identifying the 5' and 3' primer sequences for amplifying any C-terminal deletion mutant of the present invention. Moreover, the same general formulas provided above may be used in identifying the 5' and 3' primer sequences for amplifying any combination of N-terminal and C-terminal deletion mutant of the present invention. The skilled artisan would appreciate that modifications of the above nucleotide positions may be necessary for optimizing



PCR amplification.

- 5 In preferred embodiments, the following N-terminal  
 HBMYCNG deletion polypeptides are encompassed by the  
 present invention: M1-P664, T2-P664, E3-P664, K4-P664, T5-  
 P664, N6-P664, G7-P664, V8-P664, K9-P664, S10-P664, S11-  
 P664, P12-P664, A13-P664, N14-P664, N15-P664, H16-P664,  
 10 N17-P664, H18-P664, H19-P664, A20-P664, P21-P664, P22-P664,  
 A23-P664, I24-P664, K25-P664, A26-P664, N27-P664, G28-P664,  
 K29-P664, D30-P664, D31-P664, H32-P664, R33-P664, T34-P664,  
 S35-P664, S36-P664, R37-P664, P38-P664, H39-P664, S40-P664,  
 A41-P664, A42-P664, D43-P664, D44-P664, D45-P664, T46-P664,  
 15 S47-P664, S48-P664, E49-P664, L50-P664, Q51-P664, R52-P664,  
 L53-P664, A54-P664, D55-P664, V56-P664, D57-P664, A58-P664,  
 P59-P664, Q60-P664, Q61-P664, G62-P664, R63-P664, S64-P664,  
 G65-P664, F66-P664, R67-P664, R68-P664, I69-P664, V70-P664,  
 R71-P664, L72-P664, V73-P664, G74-P664, I75-P664, I76-P664,  
 20 R77-P664, E78-P664, W79-P664, A80-P664, N81-P664, K82-P664,  
 N83-P664, F84-P664, R85-P664, E86-P664, E87-P664, E88-P664,  
 P89-P664, R90-P664, P91-P664, D92-P664, S93-P664, F94-P664,  
 L95-P664, E96-P664, R97-P664, F98-P664, R99-P664, G100-  
 P664, P101-P664, E102-P664, L103-P664, Q104-P664, T105-  
 25 P664, V106-P664, T107-P664, T108-P664, Q109-P664, E110-  
 P664, G111-P664, D112-P664, G113-P664, K114-P664, G115-  
 P664, D116-P664, K117-P664, D118-P664, G119-P664, E120-  
 P664, D121-P664, K122-P664, G123-P664, T124-P664, K125-  
 P664, K126-P664, K127-P664, F128-P664, E129-P664, L130-  
 30 P664, F131-P664, V132-P664, L133-P664, D134-P664, P135-  
 P664, A136-P664, G137-P664, D138-P664, L139-P664, Y140-  
 P664, Y141-P664, C142-P664, W143-P664, L144-P664, F145-  
 P664, V146-P664, I147-P664, A148-P664, M149-P664, P150-  
 P664, V151-P664, L152-P664, Y153-P664, N154-P664, W155-  
 35 P664, C156-P664, L157-P664, L158-P664, V159-P664, A160-  
 P664, R161-P664, A162-P664, C163-P664, F164-P664, S165-  
 P664, D166-P664, L167-P664, Q168-P664, K169-P664, G170-  
 P664, Y171-P664, Y172-P664, L173-P664, V174-P664, W175-

	P664,	L176-P664,	V177-P664,	L178-P664,	D179-P664,	Y180-
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	P664,	Y186-P664,	I187-P664,	A188-P664,	D189-P664,	L190-
	P664,	F191-P664,	I192-P664,	R193-P664,	L194-P664,	R195-
	P664,	T196-P664,	G197-P664,	F198-P664,	L199-P664,	E200-
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10	P664,	K206-P664,	D207-P664,	T208-P664,	K209-P664,	K210-
	P664,	L211-P664,	R212-P664,	D213-P664,	N214-P664,	Y215-
	P664,	I216-P664,	H217-P664,	T218-P664,	L219-P664,	Q220-
	P664,	F221-P664,	K222-P664,	L223-P664,	D224-P664,	V225-
	P664,	A226-P664,	S227-P664,	I228-P664,	I229-P664,	P230-
15	P664,	T231-P664,	D232-P664,	L233-P664,	I234-P664,	Y235-
	P664,	F236-P664,	A237-P664,	V238-P664,	D239-P664,	I240-
	P664,	H241-P664,	S242-P664,	P243-P664,	E244-P664,	V245-
	P664,	R246-P664,	F247-P664,	N248-P664,	R249-P664,	L250-
	P664,	L251-P664,	H252-P664,	F253-P664,	A254-P664,	R255-
20	P664,	M256-P664,	F257-P664,	E258-P664,	F259-P664,	F260-
	P664,	D261-P664,	R262-P664,	T263-P664,	E264-P664,	T265-
	P664,	R266-P664,	T267-P664,	N268-P664,	Y269-P664,	P270-
	P664,	N271-P664,	I272-P664,	F273-P664,	R274-P664,	I275-
	P664,	S276-P664,	N277-P664,	L278-P664,	V279-P664,	L280-
25	P664,	Y281-P664,	I282-P664,	L283-P664,	V284-P664,	I285-
	P664,	I286-P664,	H287-P664,	W288-P664,	N289-P664,	A290-
	P664,	C291-P664,	I292-P664,	Y293-P664,	Y294-P664,	A295-
	P664,	I296-P664,	S297-P664,	K298-P664,	S299-P664,	I300-
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30	P664,	T306-P664,	W307-P664,	V308-P664,	Y309-P664,	P310-
	P664,	N311-P664,	I312-P664,	T313-P664,	D314-P664,	P315-
	P664,	E316-P664,	Y317-P664,	G318-P664,	Y319-P664,	L320-
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	P664,	Y326-P664,	C327-P664,	L328-P664,	Y329-P664,	W330-
35	P664,	S331-P664,	T332-P664,	L333-P664,	T334-P664,	L335-
	P664,	T336-P664,	T337-P664,	I338-P664,	G339-P664,	E340-
	P664,	T341-P664,	P342-P664,	P343-P664,	P344-P664,	V345-
	P664,	K346-P664,	D347-P664,	E348-P664,	E349-P664,	Y350-

P664, L351-P664, F352-P664, V353-P664, I354-P664, F355-  
 5 P664, D356-P664, F357-P664, L358-P664, I359-P664, G360-  
 P664, V361-P664, L362-P664, I363-P664, F364-P664, A365-  
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 10 P664, T381-P664, R382-P664, A383-P664, E384-P664, F385-  
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 15 P664, E406-P664, A407-P664, K408-P664, V409-P664, I410-  
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 P664, R426-P664, E427-P664, I428-P664, L429-P664, K430-  
 20 P664, N431-P664, L432-P664, P433-P664, A434-P664, K435-  
 P664, L436-P664, R437-P664, A438-P664, E439-P664, I440-  
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 25 P664, H456-P664, D457-P664, C458-P664, E459-P664, A460-  
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 35 P664, G506-P664, V507-P664, T508-P664, Q509-P664, Y510-  
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 P664, G516-P664, S517-P664, C518-P664, F519-P664, G520-  
 P664, E521-P664, I522-P664, S523-P664, I524-P664, L525-

- 5 P664, N526-P664, I527-P664, K528-P664, G529-P664, S530-P664, K531-P664, M532-P664, G533-P664, N534-P664, R535-P664, R536-P664, T537-P664, A538-P664, N539-P664, I540-P664, R541-P664, S542-P664, L543-P664, G544-P664, Y545-P664, S546-P664, D547-P664, L548-P664, F549-P664, C550-P664, L551-P664, S552-P664, K553-P664, D554-P664, D555-P664, L556-P664, M557-P664, E558-P664, A559-P664, V560-P664, T561-P664, E562-P664, Y563-P664, P564-P664, D565-P664, A566-P664, K567-P664, K568-P664, V569-P664, L570-P664, E571-P664, E572-P664, R573-P664, G574-P664, R575-P664, E576-P664, I577-P664, L578-P664, M579-P664, K580-P664, E581-P664, G582-P664, L583-P664, L584-P664, D585-P664, E586-P664, N587-P664, E588-P664, V589-P664, A590-P664, T591-P664, S592-P664, M593-P664, E594-P664, V595-P664, D596-P664, V597-P664, Q598-P664, E599-P664, K600-P664, L601-P664, G602-P664, Q603-P664, L604-P664, E605-P664, T606-P664, N607-P664, M608-P664, E609-P664, T610-P664, L611-P664, Y612-P664, T613-P664, R614-P664, F615-P664, G616-P664, R617-P664, L618-P664, L619-P664, A620-P664, E621-P664, Y622-P664, T623-P664, G624-P664, A625-P664, Q626-P664, Q627-P664, K628-P664, L629-P664, K630-P664, Q631-P664, R632-P664, I633-P664, T634-P664, V635-P664, L636-P664, E637-P664, T638-P664, K639-P664, M640-P664, K641-P664, Q642-P664, N643-P664, N644-P664, E645-P664, D646-P664, D647-P664, Y648-P664, L649-P664, S650-P664, D651-P664, G652-P664, M653-P664, N654-P664, S655-P664, P656-P664, E657-P664, and/or L658-P664 of SEQ ID NO:2. Polynucleotide sequences encoding these polypeptides are also provided. The present invention also encompasses the use of these N-terminal HBMYCNG deletion polypeptides as immunogenic and/or antigenic epitopes as described elsewhere herein.

In preferred embodiments, the following C-terminal HBMYCNG deletion polypeptides are encompassed by the present invention: M1-P664, M1-E663, M1-D662, M1-A661, M1-

- A660, M1-A659, M1-L658, M1-E657, M1-P656, M1-S655, M1-N654,
- 5 M1-M653, M1-G652, M1-D651, M1-S650, M1-L649, M1-Y648, M1-D647, M1-D646, M1-E645, M1-N644, M1-N643, M1-Q642, M1-K641, M1-M640, M1-K639, M1-T638, M1-E637, M1-L636, M1-V635, M1-T634, M1-I633, M1-R632, M1-Q631, M1-K630, M1-L629, M1-K628, M1-Q627, M1-Q626, M1-A625, M1-G624, M1-T623, M1-Y622, M1-
- 10 E621, M1-A620, M1-L619, M1-L618, M1-R617, M1-G616, M1-F615, M1-R614, M1-T613, M1-Y612, M1-L611, M1-T610, M1-E609, M1-M608, M1-N607, M1-T606, M1-E605, M1-L604, M1-Q603, M1-G602, M1-L601, M1-K600, M1-E599, M1-Q598, M1-V597, M1-D596, M1-V595, M1-E594, M1-M593, M1-S592, M1-T591, M1-A590, M1-V589,
- 15 M1-E588, M1-N587, M1-E586, M1-D585, M1-L584, M1-L583, M1-G582, M1-E581, M1-K580, M1-M579, M1-L578, M1-I577, M1-E576, M1-R575, M1-G574, M1-R573, M1-E572, M1-E571, M1-L570, M1-V569, M1-K568, M1-K567, M1-A566, M1-D565, M1-P564, M1-Y563, M1-E562, M1-T561, M1-V560, M1-A559, M1-E558, M1-M557, M1-
- 20 L556, M1-D555, M1-D554, M1-K553, M1-S552, M1-L551, M1-C550, M1-F549, M1-L548, M1-D547, M1-S546, M1-Y545, M1-G544, M1-L543, M1-S542, M1-R541, M1-I540, M1-N539, M1-A538, M1-T537, M1-R536, M1-R535, M1-N534, M1-G533, M1-M532, M1-K531, M1-S530, M1-G529, M1-K528, M1-I527, M1-N526, M1-L525, M1-I524,
- 25 M1-S523, M1-I522, M1-E521, M1-G520, M1-F519, M1-C518, M1-S517, M1-G516, M1-A515, M1-S514, M1-L513, M1-L512, M1-A511, M1-Y510, M1-Q509, M1-T508, M1-V507, M1-G506, M1-D505, M1-D504, M1-A503, M1-V502, M1-V501, M1-A500, M1-L499, M1-K498, M1-G497, M1-E496, M1-K495, M1-I494, M1-I493, M1-Y492, M1-
- 30 M491, M1-E490, M1-K489, M1-G488, M1-I487, M1-D486, M1-G485, M1-K484, M1-R483, M1-C482, M1-I481, M1-Y480, M1-D479, M1-G478, M1-P477, M1-S476, M1-F475, M1-V474, M1-Q473, M1-P472, M1-R471, M1-L470, M1-K469, M1-L468, M1-V467, M1-L466, M1-E465, M1-V464, M1-L463, M1-L462, M1-G461, M1-A460, M1-E459,
- 35 M1-C458, M1-D457, M1-H456, M1-F455, M1-I454, M1-R453, M1-V452, M1-K451, M1-K450, M1-L449, M1-T448, M1-S447, M1-L446, M1-H445, M1-V444, M1-N443, M1-T442, M1-A441, M1-I440, M1-E439, M1-A438, M1-R437, M1-L436, M1-K435, M1-A434, M1-P433,

M1-L432, M1-N431, M1-K430, M1-L429, M1-I428, M1-E427, M1-  
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 M1-N419, M1-T418, M1-W417, M1-L416, M1-Y415, M1-D414, M1-  
 F413, M1-W412, M1-R411, M1-I410, M1-V409, M1-K408, M1-A407,  
 M1-E406, M1-M405, M1-G404, M1-K403, M1-S402, M1-V401, M1-  
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 10 M1-K393, M1-V392, M1-A391, M1-D390, M1-I389, M1-K388, M1-  
 A387, M1-Q386, M1-F385, M1-E384, M1-A383, M1-R382, M1-T381,  
 M1-A380, M1-N379, M1-M378, M1-N377, M1-S376, M1-I375, M1-  
 M374, M1-S373, M1-G372, M1-V371, M1-N370, M1-G369, M1-V368,  
 M1-I367, M1-T366, M1-A365, M1-F364, M1-I363, M1-L362, M1-  
 15 V361, M1-G360, M1-I359, M1-L358, M1-F357, M1-D356, M1-F355,  
 M1-I354, M1-V353, M1-F352, M1-L351, M1-Y350, M1-E349, M1-  
 E348, M1-D347, M1-K346, M1-V345, M1-P344, M1-P343, M1-P342,  
 M1-T341, M1-E340, M1-G339, M1-I338, M1-T337, M1-T336, M1-  
 L335, M1-T334, M1-L333, M1-T332, M1-S331, M1-W330, M1-Y329,  
 20 M1-L328, M1-C327, M1-Y326, M1-I325, M1-Y324, M1-E323, M1-  
 R322, M1-A321, M1-L320, M1-Y319, M1-G318, M1-Y317, M1-E316,  
 M1-P315, M1-D314, M1-T313, M1-I312, M1-N311, M1-P310, M1-  
 Y309, M1-V308, M1-W307, M1-T306, M1-D305, M1-V304, M1-G303,  
 M1-F302, M1-G301, M1-I300, M1-S299, M1-K298, M1-S297, M1-  
 25 I296, M1-A295, M1-Y294, M1-Y293, M1-I292, M1-C291, M1-A290,  
 M1-N289, M1-W288, M1-H287, M1-I286, M1-I285, M1-V284, M1-  
 L283, M1-I282, M1-Y281, M1-L280, M1-V279, M1-L278, M1-N277,  
 M1-S276, M1-I275, M1-R274, M1-F273, M1-I272, M1-N271, M1-  
 P270, M1-Y269, M1-N268, M1-T267, M1-R266, M1-T265, M1-E264,  
 30 M1-T263, M1-R262, M1-D261, M1-F260, M1-F259, M1-E258, M1-  
 F257, M1-M256, M1-R255, M1-A254, M1-F253, M1-H252, M1-L251,  
 M1-L250, M1-R249, M1-N248, M1-F247, M1-R246, M1-V245, M1-  
 E244, M1-P243, M1-S242, M1-H241, M1-I240, M1-D239, M1-V238,  
 M1-A237, M1-F236, M1-Y235, M1-I234, M1-L233, M1-D232, M1-  
 35 T231, M1-P230, M1-I229, M1-I228, M1-S227, M1-A226, M1-V225,  
 M1-D224, M1-L223, M1-K222, M1-F221, M1-Q220, M1-L219, M1-  
 T218, M1-H217, M1-I216, M1-Y215, M1-N214, M1-D213, M1-R212,  
 M1-L211, M1-K210, M1-K209, M1-T208, M1-D207, M1-K206, M1-

V205, M1-L204, M1-L203, M1-G202, M1-Q201, M1-E200, M1-L199,  
 5 M1-F198, M1-G197, M1-T196, M1-R195, M1-L194, M1-R193, M1-  
 I192, M1-F191, M1-L190, M1-D189, M1-A188, M1-I187, M1-Y186,  
 M1-V185, M1-V184, M1-D183, M1-S182, M1-V181, M1-Y180, M1-  
 D179, M1-L178, M1-V177, M1-L176, M1-W175, M1-V174, M1-L173,  
 M1-Y172, M1-Y171, M1-G170, M1-K169, M1-Q168, M1-L167, M1-  
 10 D166, M1-S165, M1-F164, M1-C163, M1-A162, M1-R161, M1-A160,  
 M1-V159, M1-L158, M1-L157, M1-C156, M1-W155, M1-N154, M1-  
 Y153, M1-L152, M1-V151, M1-P150, M1-M149, M1-A148, M1-I147,  
 M1-V146, M1-F145, M1-L144, M1-W143, M1-C142, M1-Y141, M1-  
 Y140, M1-L139, M1-D138, M1-G137, M1-A136, M1-P135, M1-D134,  
 15 M1-L133, M1-V132, M1-F131, M1-L130, M1-E129, M1-F128, M1-  
 K127, M1-K126, M1-K125, M1-T124, M1-G123, M1-K122, M1-D121,  
 M1-E120, M1-G119, M1-D118, M1-K117, M1-D116, M1-G115, M1-  
 K114, M1-G113, M1-D112, M1-G111, M1-E110, M1-Q109, M1-T108,  
 M1-T107, M1-V106, M1-T105, M1-Q104, M1-L103, M1-E102, M1-  
 20 P101, M1-G100, M1-R99, M1-F98, M1-R97, M1-E96, M1-L95, M1-  
 F94, M1-S93, M1-D92, M1-P91, M1-R90, M1-P89, M1-E88, M1-  
 E87, M1-E86, M1-R85, M1-F84, M1-N83, M1-K82, M1-N81, M1-  
 A80, M1-W79, M1-E78, M1-R77, M1-I76, M1-I75, M1-G74, M1-  
 V73, M1-L72, M1-R71, M1-V70, M1-I69, M1-R68, M1-R67, M1-  
 25 F66, M1-G65, M1-S64, M1-R63, M1-G62, M1-Q61, M1-Q60, M1-  
 P59, M1-A58, M1-D57, M1-V56, M1-D55, M1-A54, M1-L53, M1-  
 R52, M1-Q51, M1-L50, M1-E49, M1-S48, M1-S47, M1-T46, M1-  
 D45, M1-D44, M1-D43, M1-A42, M1-A41, M1-S40, M1-H39, M1-  
 P38, M1-R37, M1-S36, M1-S35, M1-T34, M1-R33, M1-H32, M1-  
 30 D31, M1-D30, M1-K29, M1-G28, M1-N27, M1-A26, M1-K25, M1-  
 I24, M1-A23, M1-P22, M1-P21, M1-A20, M1-H19, M1-H18, M1-  
 N17, M1-H16, M1-N15, M1-N14, M1-A13, M1-P12, M1-S11, M1-  
 S10, M1-K9, M1-V8, and/or M1-G7 of SEQ ID NO:2.  
 Polynucleotide sequences encoding these polypeptides are  
 also provided. The present invention also encompasses the  
 use of these C-terminal HBMYCNG deletion polypeptides as  
 immunogenic and/or antigenic epitopes as described  
 elsewhere herein.

The present invention also encompasses the the same N-  
5 and/or C-terminal deletion mutants for the varant HBMYCNG  
polypeptide depicted in FIG. 6 (SEQ ID NO:24) with the  
appropriate amino acid and encoding nucleic acid  
substitutions. Methods of substituting such sequences are  
known in the art.

10

6.6 Method Of Enhancing The Biological Activity/Functional  
Characteristics Of Invention Through Molecular Evolution.

Although many of the most biologically active proteins  
known are highly effective for their specified function in  
15 an organism, they often possess characteristics that make  
them undesirable for transgenic, therapeutic,  
pharmaceutical, and/or industrial applications. Among these  
traits, a short physiological half-life is the most  
prominent problem, and is present either at the level of  
20 the protein, or the level of the proteins mRNA. The ability  
to extend the half-life, for example, would be particularly  
important for a proteins use in gene therapy, transgenic  
animal production, the bioprocess production and  
purification of the protein, and use of the protein as a  
25 chemical modulator among others. Therefore, there is a need  
to identify novel variants of isolated proteins possessing  
characteristics which enhance their application as a  
therapeutic for treating diseases of animal origin, in  
addition to the proteins applicability to common industrial  
and pharmaceutical applications.

30 Thus, one aspect of the present invention relates to  
the ability to enhance specific characteristics of  
invention through directed molecular evolution. Such an  
enhancement may, in a non-limiting example, benefit the  
35 inventions utility as an essential component in a kit, the  
inventions physical attributes such as its solubility,  
structure, or codon optimization, the inventions specific  
biological activity, including any associated enzymatic



activity, the proteins enzyme kinetics, the proteins  $K_i$ ,  
 5  $K_{cat}$ ,  $K_m$ ,  $V_{max}$ ,  $K_d$ , protein-protein activity, protein-DNA  
 binding activity, antagonist/inhibitory activity (including  
 direct or indirect interaction), agonist activity  
 (including direct or indirect interaction), the proteins  
 antigenicity (e.g., where it would be desirable to either  
 10 increase or decrease the antigenic potential of the  
 protein), the immunogenicity of the protein, the ability of  
 the protein to form dimers, trimers, or multimers with  
 either itself or other proteins, the antigenic efficacy of  
 the invention, including its subsequent use a preventative  
 15 treatment for disease or disease states, or as an effector  
 for targeting diseased genes. Moreover, the ability to  
 enhance specific characteristics of a protein may also be  
 applicable to changing the characterized activity of an  
 enzyme to an activity completely unrelated to its initially  
 20 characterized activity. Other desirable enhancements of the  
 invention would be specific to each individual protein, and  
 would thus be well known in the art and contemplated by the  
 present invention.

For example, an engineered ion channel protein may be  
 25 constitutively active upon binding of its cognate ligand.  
 Alternatively, an engineered ion channel protein may be  
 constitutively active in the absence of ligand binding. In  
 yet another example, an engineered ion channel protein may  
 be capable of being activated with less than all of the  
 30 regulatory factors and/or conditions typically required for  
 ion channel protein activation (e.g., ion flux, ligand  
 binding, phosphorylation, conformational changes, etc.).  
 Such ion channel protein would be useful in screens to  
 identify ion channel protein modulators, among other uses  
 35 described herein.

Directed evolution is comprised of several steps. The  
 first step is to establish a library of variants for the  
 gene or protein of interest. The most important step is to

then select for those variants that entail the activity you  
 5 wish to identify. The design of the screen is essential  
 since your screen should be selective enough to eliminate  
 non-useful variants, but not so stringent as to eliminate  
 all variants. The last step is then to repeat the above  
 steps using the best variant from the previous screen. Each  
 10 successive cycle, can then be tailored as necessary, such  
 as increasing the stringency of the screen, for example.

Over the years, there have been a number of methods  
 developed to introduce mutations into macromolecules. Some  
 of these methods include, random mutagenesis, "error-prone"  
 15 PCR, chemical mutagenesis, site-directed mutagenesis, and  
 other methods well known in the art (for a comprehensive  
 listing of current mutagenesis methods, see Maniatis,  
 Molecular Cloning: A Laboratory Manual, Cold Spring Harbor  
 Press, Cold Spring, NY (1982)). Typically, such methods  
 20 have been used, for example, as tools for identifying the  
 core functional region(s) of a protein or the function of  
 specific domains of a protein (if a multi-domain protein).  
 However, such methods have more recently been applied to  
 the identification of macromolecule variants with specific  
 25 or enhanced characteristics.

Random mutagenesis has been the most widely recognized  
 method to date. Typically, this has been carried out either  
 through the use of "error-prone" PCR (as described in  
 Moore, J., et al, Nature Biotechnology 14:458, (1996), or  
 30 through the application of randomized synthetic  
 oligonucleotides corresponding to specific regions of  
 interest (as described by Derbyshire, K.M. et al, Gene,  
 46:145-152, (1986), and Hill, DE, et al, Methods Enzymol.,  
 55:559-568, (1987)). Both approaches have limits to the  
 35 level of mutagenesis that can be obtained. However, either  
 approach enables the investigator to effectively control  
 the rate of mutagenesis. This is particularly important  
 considering the fact that mutations beneficial to the

activity of the enzyme are fairly rare. In fact, using too  
 5 high a level of mutagenesis may counter or inhibit the  
 desired benefit of a useful mutation.

While both of the aforementioned methods are effective  
 for creating randomized pools of macromolecule variants, a  
 third method, termed "DNA Shuffling", or "sexual PCR" (WPC,  
 10 Sterner, PNAS, 91:10747, (1994)) has recently been  
 elucidated. DNA shuffling has also been referred to as  
 "directed molecular evolution", "exon-shuffling", "directed  
 enzyme evolution", "in vitro evolution", and "artificial  
 evolution". Such reference terms are known in the art and  
 15 are encompassed by the invention. This new, preferred,  
 method apparently overcomes the limitations of the previous  
 methods in that it not only propagates positive traits, but  
 simultaneously eliminates negative traits in the resulting  
 progeny.

20 DNA shuffling accomplishes this task by combining the  
 principal of in vitro recombination, along with the method  
 of "error-prone" PCR. In effect, you begin with a randomly  
 digested pool of small fragments of your gene, created by  
 Dnase I digestion, and then introduce said random fragments  
 25 into an "error-prone" PCR assembly reaction. During the PCR  
 reaction, the randomly sized DNA fragments not only  
 hybridize to their cognate strand, but also may hybridize  
 to other DNA fragments corresponding to different regions  
 of the polynucleotide of interest - regions not typically  
 30 accessible via hybridization of the entire polynucleotide.  
 Moreover, since the PCR assembly reaction utilizes "error-  
 prone" PCR reaction conditions, random mutations are  
 introduced during the DNA synthesis step of the PCR  
 reaction for all of the fragments -further diversifying the  
 35 potential hybridization sites during the annealing step of  
 the reaction.

A variety of reaction conditions could be utilized to  
 carry-out the DNA shuffling reaction. However, specific

reaction conditions for DNA shuffling are provided, for  
 5 example, in PNAS, 91:10747, (1994). Briefly:

Prepare the DNA substrate to be subjected to the DNA  
 shuffling reaction. Preparation may be in the form of  
 simply purifying the DNA from contaminating cellular  
 material, chemicals, buffers, oligonucleotide primers,  
 10 deoxynucleotides, RNAs, etc., and may entail the use of DNA  
 purification kits as those provided by Qiagen, Inc., or by  
 the Promega, Corp., for example.

Once the DNA substrate has been purified, it would be  
 subjected to Dnase I digestion. About 2-4ug of the DNA  
 15 substrate(s) would be digested with .0015 units of Dnase I  
 (Sigma) per ul in 100ul of 50mM Tris-HCL, pH 7.4/1mM MgCl<sub>2</sub>  
 for 10-20 min. at room temperature. The resulting fragments  
 of 10-50bp could then be purified by running them through  
 a 2% low-melting point agarose gel by electrophoresis onto  
 20 DE81 ion-exchange paper (Whatman) or could be purified  
 using Microcon concentrators (Amicon) of the appropriate  
 molecular weight cutoff, or could use oligonucleotide  
 purification columns (Qiagen), in addition to other methods  
 known in the art. If using DE81 ion-exchange paper, the 10-  
 25 50bp fragments could be eluted from said paper using 1M  
 NaCl, followed by ethanol precipitation.

The resulting purified fragments would then be  
 subjected to a PCR assembly reaction by re-suspension in a  
 PCR mixture containing: 2mM of each dNTP, 2.2mM MgCl<sub>2</sub>, 50  
 30 mM KCl, 10mM Tris•HCL, pH 9.0, and 0.1% Triton X-100, at a  
 final fragment concentration of 10-30ng/ul. No primers are  
 added at this point. *Taq* DNA polymerase (Promega) would be  
 used at 2.5 units per 100ul of reaction mixture. A PCR  
 program of 94 C for 60s; 94 C for 30s, 50-55 C for 30s, and  
 35 72 C for 30s using 30-45 cycles, followed by 72 C for 5min  
 using an MJ Research (Cambridge, MA) PTC-150 thermocycler.  
 After the assembly reaction is completed, a 1:40 dilution  
 of the resulting primerless product would then be

introduced into a PCR mixture (using the same buffer  
5 mixture used for the assembly reaction) containing 0.8um of  
each primer and subjecting this mixture to 15 cycles of PCR  
(using 94 C for 30s, 50 C for 30s, and 72 C for 30s). The  
referred primers would be primers corresponding to the  
nucleic acid sequences of the polynucleotide(s) utilized in  
10 the shuffling reaction. Said primers could consist of  
modified nucleic acid base pairs using methods known in the  
art and referred to else where herein, or could contain  
additional sequences (i.e., for adding restriction sites,  
mutating specific base-pairs, etc.).

15 The resulting shuffled, assembled, and amplified  
product can be purified using methods well known in the art  
(e.g., Qiagen PCR purification kits) and then subsequently  
cloned using appropriate restriction enzymes.

Although a number of variations of DNA shuffling have  
20 been published to date, such variations would be obvious to  
the skilled artisan and are encompassed by the invention.  
The DNA shuffling method can also be tailored to the  
desired level of mutagenesis using the methods described by  
Zhao, et al. (Nucl Acid Res., 25(6):1307-1308, (1997)).

25 As described above, once the randomized pool has been  
created, it can then be subjected to a specific screen to  
identify the variant possessing the desired  
characteristic(s). Once the variant has been identified,  
DNA corresponding to the variant could then be used as the  
30 DNA substrate for initiating another round of DNA  
shuffling. This cycle of shuffling, selecting the optimized  
variant of interest, and then re-shuffling, can be repeated  
until the ultimate variant is obtained. Examples of model  
screens applied to identify variants created using DNA  
35 shuffling technology may be found in the following  
publications: J. C., Moore, et al., J. Mol. Biol., 272:336-  
347, (1997), F.R., Cross, et al., Mol. Cell. Biol.,  
18:2923-2931, (1998), and A. Cramerl., et al., Nat.

Biotech., 15:436-438, (1997).

5 DNA shuffling has several advantages. First, it makes use of beneficial mutations. When combined with screening, DNA shuffling allows the discovery of the best mutational combinations and does not assume that the best combination contains all the mutations in a population. Secondly,  
10 recombination occurs simultaneously with point mutagenesis. An effect of forcing DNA polymerase to synthesize full-length genes from the small fragment DNA pool is a background mutagenesis rate. In combination with a stringent selection method, enzymatic activity has been  
15 evolved up to 16000 fold increase over the wild-type form of the enzyme. In essence, the background mutagenesis yielded the genetic variability on which recombination acted to enhance the activity.

A third feature of recombination is that it can be  
20 used to remove deleterious mutations. As discussed above, during the process of the randomization, for every one beneficial mutation, there may be at least one or more neutral or inhibitory mutations. Such mutations can be removed by including in the assembly reaction an excess of  
25 the wild-type random-size fragments, in addition to the random-size fragments of the selected mutant from the previous selection. During the next selection, some of the most active variants of the polynucleotide/  
polypeptide/enzyme, should have lost the inhibitory  
30 mutations.

Finally, recombination enables parallel processing. This represents a significant advantage since there are likely multiple characteristics that would make a protein more desirable (e.g. solubility, activity, etc.). Since it  
35 is increasingly difficult to screen for more than one desirable trait at a time, other methods of molecular evolution tend to be inhibitory. However, using recombination, it would be possible to combine the

randomized fragments of the best representative variants  
5 for the various traits, and then select for multiple  
properties at once.

DNA shuffling can also be applied to the  
polynucleotides and polypeptides of the present invention  
to decrease their immunogenicity in a specified host,  
10 particularly if the polynucleotides and polypeptides  
provide a therapeutic use. For example, a particular  
variant of the present invention may be created and  
isolated using DNA shuffling technology. Such a variant may  
have all of the desired characteristics, though may be  
15 highly immunogenic in a host due to its novel intrinsic  
structure. Specifically, the desired characteristic may  
cause the polypeptide to have a non-native structure which  
could no longer be recognized as a "self" molecule, but  
rather as a "foreign", and thus activate a host immune  
20 response directed against the novel variant. Such a  
limitation can be overcome, for example, by including a  
copy of the gene sequence for a xenobiotic ortholog of the  
native protein in with the gene sequence of the novel  
variant gene in one or more cycles of DNA shuffling. The  
25 molar ratio of the ortholog and novel variant DNAs could be  
varied accordingly. Ideally, the resulting hybrid variant  
identified would contain at least some of the coding  
sequence which enabled the xenobiotic protein to evade the  
host immune system, and additionally, the coding sequence  
30 of the original novel variant that provided the desired  
characteristics.

Likewise, the invention encompasses the application of  
DNA shuffling technology to the evolution of  
polynucleotides and polypeptides of the invention, wherein  
35 one or more cycles of DNA shuffling include, in addition to  
the gene template DNA, oligonucleotides coding for known  
allelic sequences, optimized codon sequences, known variant  
sequences, known polynucleotide polymorphism sequences,

known ortholog sequences, known homolog sequences,  
5 additional homologous sequences, additional non-homologous  
sequences, sequences from another species, and any number  
and combination of the above.

In addition to the described methods above, there are  
a number of related methods that may also be applicable, or  
10 desirable in certain cases. Representative among these are  
the methods discussed in PCT applications WO 98/31700, and  
WO 98/32845, which are hereby incorporated by reference.  
Furthermore, related methods can also be applied to the  
polynucleotide sequences of the present invention in order  
15 to evolve invention for creating ideal variants for use in  
gene therapy, protein engineering, evolution of whole cells  
containing the variant, or in the evolution of entire  
enzyme pathways containing polynucleotides of the invention  
as described in PCT applications WO 98/13485, WO 98/13487,  
20 WO 98/27230, WO 98/31837, and Crameri, A., et al., Nat.  
Biotech., 15:436-438, (1997), respectively.

Additional methods of applying "DNA Shuffling"  
technology to the polynucleotides and polypeptides of the  
present invention, including their proposed applications,  
25 may be found in US Patent No. 5,605,793; PCT Application  
No. WO 95/22625; PCT Application No. WO 97/20078; PCT  
Application No. WO 97/35966; and PCT Application No. WO  
98/42832; PCT Application No. The forgoing are hereby  
incorporated in their entirety herein for all purposes.

30 7. DEPOSIT OF MICROORGANISMS

The following microorganisms were deposited with the  
American Type Culture Collection (ATCC), 10801 University  
Blvd., Manassas, Virginia 20110 on \_\_\_\_\_ and  
35 assigned the following numbers:

Microorganism  
HBMYCNG-pcDNA

ATCC Deposit No.  
\_\_\_\_\_



5           The present invention is not to be limited in scope  
by the specific embodiments described herein, which are  
intended as single illustrations of individual aspects of  
the invention, and functionally equivalent methods and  
components are within the scope of the invention. Indeed,  
10 various modifications of the invention, in addition to  
those shown and described herein will become apparent to  
those skilled in the art from the foregoing description  
and accompanying drawings. Such modifications are  
intended to fall within the scope of the appended claims.

15           Various publications are cited herein, the  
disclosures of which are incorporated by reference in  
their entireties.

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